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The Journal of Clinical Investigation

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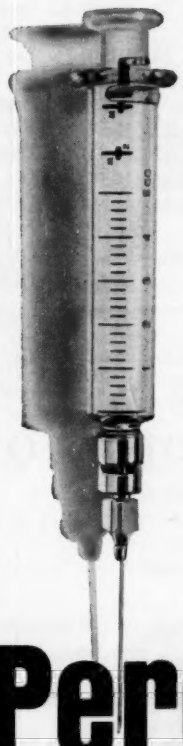

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Activité de certaines protéines de la coagulation dans le plasma et les urines au cours du syndrôme néphrotique. H. YATZIDIS et G. RICHET.

Facteurs de l'hyperaldolasémie au cours des myopathies. F. SCHAPIRA, J. DEMOS, G. SCHAPIRA et J. C. DREYFUS.

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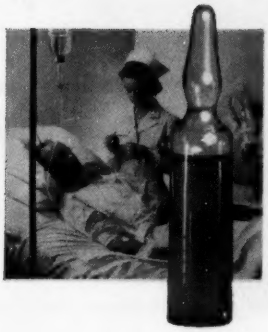

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Measurement of the Ventilation-Perfusion Ratio Inequality in the Lung by the Analysis of a Single Expirate. By J. B. WEST, K. T. FOWLER, P. HUGH-JONES and T. V. O'DONNELL.

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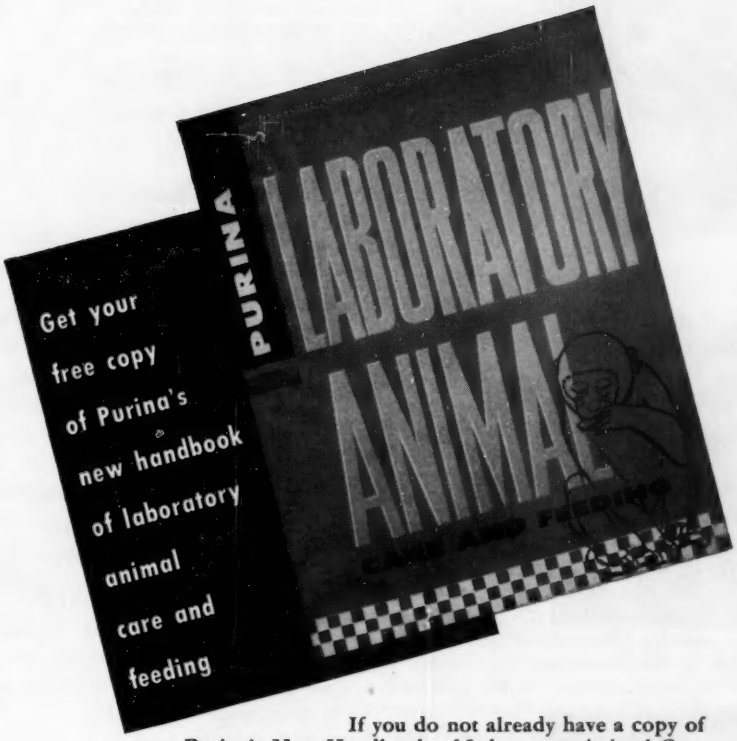

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**_Ibid._, p. 600.

EXCHANGE OF CERULOPLASMIN COPPER WITH IONIC Cu^{64} WITH REFERENCE TO WILSON'S DISEASE [1]

By I. HERBERT SCHEINBERG and ANATOL G. MORELL

(From the Department of Medicine, Albert Einstein College of Medicine and the Bronx Municipal Hospital Center, New York, N. Y.)

(Submitted for publication January 28, 1957; accepted March 29, 1957)

Ceruloplasmin is a blue alpha-globulin which is normally present in human plasma at a concentration of 15 to 30 mg. per ml. (1–4). This protein contains 8 atoms of copper per molecule, and has a molecular weight of 150,000 (1). Ceruloplasmin's physiological function is unknown although its plasma level rises in various diseases and in pregnancy (3, 5–7), and is lower than normal in patients with hepatolenticular degeneration (Wilson's disease) (2, 3, 8), the nephrotic syndrome (3), and in the newborn infant (6). In the nephrotic syndrome, the low level of ceruloplasmin is apparently due to urinary loss of ceruloplasmin (3). In Wilson's disease, however, the deficiency or absence of the protein is probably the consequence of a specific, inherited inability to synthesize ceruloplasmin at a normal rate (9). Several groups of investigators believe that the primary abnormality of Wilson's disease is a deficiency of ceruloplasmin which leads, by an unknown mechanism, to excessive net absorption of dietary copper (2, 10–13).

In studies on the chemistry of ceruloplasmin, attempts have been made to remove reversibly its tightly bound copper, but these have been unsuccessful (14, 15). Nor have experiments demonstrating exchange of radioactive ionic copper with the copper in ceruloplasmin been reported. Radioactive ionic copper, however, has been exchanged with the tightly bound copper of another protein, ascorbic acid oxidase, which shows some resemblances to ceruloplasmin (16–18). The aim of the experiments reported in this paper was to determine if exchange could be effected between ceruloplasmin copper and ionic copper. The results obtained indicate that such an exchange can occur under certain conditions.

[1] This work was supported in part by a grant (A-1059) from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, United States Public Health Service, and by a grant (NONR-1765 [00]) from the Office of Naval Research.

METHOD

Experimental Procedure

The design of the experiment was as follows. A stock solution (Solution 1) was made which contained 47.0 mcg. per ml. of ionic copper, labelled with $copper^{64}$, and 47.0 mcg. per ml. of ceruloplasmin copper. A 2.0-ml. aliquot of this solution was diluted to 25.0 ml., and served as a control (Solution 2) which contained all the ceruloplasmin, radioactive copper, and free ionic copper used in preparing Solutions 3 and 4. To a second 2.0-ml. aliquot, 20 mg. of ascorbic acid was added, after which all the ionic copper, but not the ceruloplasmin copper, was removed by ion exchange resins, and dilution to 25.0 ml. was made (Solution 3). Another solution was prepared exactly as Solution 3, except that no ascorbic acid was used (Solution 4). Solution 5 was prepared in identical fashion from a solution which contained the same amount of labelled ionic copper and ascorbic acid as 2.0 ml. of Solution 1, but no ceruloplasmin. The entire experiment was done in duplicate, providing an A and B set of solutions. Solutions 2, 3, 4 and 5 were analyzed for radioactive copper, free ionic copper, total copper, and ceruloplasmin, except that Solution 5 obviously contained no ceruloplasmin. The presence in a solution of radioactivity and ceruloplasmin in the absence of appreciable free ionic copper indicated that exchange had occurred.

Apparatus

Beckman DU Spectrophotometer modified for use with a) cuvettes of 5.0-cm. path-length and 2.5-ml. volume, and b) thermospacers for maintaining solutions at constant temperature.

Gas-flow radiation detector and scaler (Nuclear Instrument and Chemical Corp., Model Nos. D-47 and 161-A).

Materials

Ceruloplasmin. Ceruloplasmin was prepared from Fraction IV of Cohn (19) by fractionation using methanol and chloroform.[2] The preparation used, AK-38S,

[2] Fraction IV was obtained through the kindness of Dr. James A. McComb, Director, Dr. John M. Newell, and Mr. Lewis H. Larsen of the Massachusetts Public Health Biologic Laboratories, and of Dr. Sam T. Gibson, Director, of the American National Red Cross Blood Program. The preparation of the ceruloplasmin was carried out by Dr. H. O. Singher and Dr. Alan Keltz of the Ortho Research Foundation, Raritan, New Jersey.

TABLE I

Ceruloplasmin, Preparation AK-38S

1 Total protein*	2 Ceruloplasmin†	3 Ceruloplasmin as % of total protein	4 Total copper‡	5 Free copper§	6 Ceruloplasmin copper‖	7 Calculated ceruloplasmin copper¶	8 Ratio of ΔO.D. at λ to ΔO.D. at 610 mμ			
mg./ml.	*mg./ml.*		*mcg./ml.*	*mcg./ml.*	*mcg./ml.*	*mcg./ml.*				
17.1	14.3	83.6	49.6±0.9	2.6±.14	47.1	48.6				
							λ mμ:	*540*	*580*	*610*	*660*
							AK-38S:	0.36	0.82	1.00	0.75
							H. and L.:**	0.37	0.85	1.00	0.68

* Nitrogen content × 6.67.
† $\Delta O.D._{610}^{1\,cm.} \div 0.34$.
‡ Wet digestion. See methods. Average of five determinations.
§ See methods.
‖ Ceruloplasmin copper = (Column 4) − (Column 5).
¶ (Column 2) × copper content of ceruloplasmin = (14.3) (0.0034).
** H. and L. values read from Figure 1 of Reference 1.

was a clear-blue solution which had been sterilized by Seitz filtration. Table I gives some characteristics of this preparation together with a comparison of its absorption spectrum with that given by ceruloplasmin prepared by Holmberg and Laurell (1). AK-38S had oxidase activity toward paraphenylenediamine, as shown below. If allowed to stand at about 2° C. at pH 4.8, platelike crystals of ceruloplasmin precipitated out of solution, although the yield was so small that crystallization was not used preparatively.

Copper^{64}. Copper^{64} acetate was obtained from Abbott Laboratories, Oak Ridge, Tennessee. The preparation used for the experiments reported in this paper contained 3.2 mc. per mg. at the time of shipment from Oak Ridge. Its concentration, determined in triplicate, was 2.17 mg. of copper per ml. Dilutions were made quantitatively with sufficient stable copper sulfate to produce both the desired number of counts per minute (c.p.m.) and final copper concentration.

For determination of copper^{64}, duplicate 1-ml. aliquots of the solutions to be analyzed were dried on aluminum planchettes and counted. All solutions, except Solution 5, had almost the same buffer and protein content so that absorption of β-particles by salt and protein was roughly equal in each sample which was radioactive. All counts were corrected for decay of radioactivity, using a half-life of 12.8 hours, and for background, which never exceeded 16 c.p.m. Proper operation of the counting equipment was controlled daily by counting a carbon^{14} standard, and determining the background radioactivity.

Resins. The cation exchange resin, Amberlite IR-120(H), Analytical Grade (Rohm and Haas Co., Philadelphia, Pa.), was washed in a column with water and then, successively, with twice its volume of 5 per cent HCl, four volumes of water, eight volumes of 5 per cent NaCl and eight volumes of water. This entire cycle was repeated a second time, using eight volumes of 5 per cent HCl instead of two. The resin was finally washed with water overnight so that the effluent pH was 6.4 (16). This resin, now on the sodium cycle, was analyzed for total copper by the method described below and found to contain 0.26 mcg. of copper per ml. of resin.

The chloride cycle of the anion exchange resin, Amberlite IRA-400 (Rohm and Haas), was converted quantitatively to the OH form by passing over it, in a column, approximately 60 volumes of 6 per cent sodium hydroxide followed by 2 volumes of 6 per cent carbonate-free potassium hydroxide, after which the effluent was free of chloride. The resin was then washed with about 15 volumes of water so that the effluent was no longer alkaline to litmus and put on the acetate cycle by adding 5 per cent acetic acid to it in a beaker until the supernatant solution stayed acid, after stirring, to litmus. The resin was washed with distilled water to neutrality. Analysis, after wet digestion, showed it to contain 1.0 mcg. of copper per ml.

Resin columns were made in 90 × 1 cm. (external diameter) lengths of glass tubing. As used, the resin columns consisted, successively, from top to bottom, of 10 ml. of anion exchange (acetate) resin, 20 ml. of cation exchange resin (sodium), 10 ml. of acetate resin, 10 ml. of sodium resin, and a loose plug of acid-washed glass wool. Each column was washed with 200 ml. of pH 5.8, $\Gamma/2 = 0.1$ acetate buffer before use. Solutions were passed through the columns at room temperature at 2 to 4 drops per minute. Each column was used only once. In each column the ratio of sodium-cycle-cation-exchange-resin to ionic copper in Solutions 3, 4 and 5, before "columnization," was always 2 ml. resin per 3 mcg. of copper. Such a column would be expected to effect complete removal of ionic copper since in the experiments of Joselow and Dawson half as much of a very similar cation-exchange resin (Amberlite IR-100), treated and used in almost the same manner, removed all traces of ionic copper (16). The acetate-cycle-anion-exchange-resin was added to the columns in order to remove ascorbic acid which changes the blue color, enzymatic activity and light absorption at 280 mμ of solutions of ceruloplasmin.

Quantitative determination of ceruloplasmin—Spectrophotometric

This method, slightly modified from that of Holmberg and Laurell (1), was applied, in the studies to be reported below, only to purified ceruloplasmin fractions of plasma. With slight changes, to be reported in detail in a subsequent paper, the method is applicable to citrated plasma itself, although for unknown reasons it will not work with serum. This analysis depends on the blue color of ceruloplasmin, since measurement of the optical density (O.D.) of the solution at 610 mμ, before and after destruction of the blue color, allows the concentration of ceruloplasmin to be calculated from its extinction coefficient.

Procedure. Using 5.0-cm. path-length cuvettes, the optical density of the solution to be analyzed is measured in the Beckman Spectrophotometer at about six wavelengths between 540 and 660 mμ including 610 mμ. After adding 30 mg. of ascorbic acid crystals to the contents of the cuvette, and mixing, optical densities are measured again at the same wavelengths when the optical density at 610 mμ has fallen to a minimum. The differences in optical density (ΔO.D.) before and after addition of the ascorbic acid, plotted against wavelength, should give a curve with a maxium at about 605 to 620 mμ similar to that found by Holmberg and Laurell (Table I). The concentration of ceruloplasmin in mg. per ml. is found by dividing $\Delta O.D._{.610}$ by the extinction coefficient, which is 0.34, for a 1 mg. per ml. solution in a cell of 5-cm. path-length.[3]

The standard deviation of results obtained by this method, calculated from seven sets of duplicate determinations, was 0.0072 mg. ceruloplasmin per ml. The accuracy can be estimated from the results in Table I where ceruloplasmin copper, calculated in Column 7 as the product of the ceruloplasmin concentration, measured by this method, and the content of copper in ceruloplasmin (1) agreed with the independently determined ceruloplasmin copper given in Column 6 within 3 per cent.

Quantitative determination of ceruloplasmin—Enzymatic

A method of quantitatively measuring the oxidase activity of ceruloplasmin is of value in determining whether the protein has been enzymatically inactivated by the experimental procedures. This measurement was of importance since ascorbic acid oxidase appears to be inactivated when it functions enzymatically (16). The following method was developed to measure this activity spectrophotometrically by allowing ceruloplasmin to catalyze the oxidation of paraphenylenediamine (ppd) to colored compounds at 30.0° C. in a Beckman Spectrophotometer.

Use of this method depends on the assumptions that 1) ceruloplasmin catalyzes the oxidation of ppd, 2) there is sufficient dissolved oxygen in the solution not to limit the rate of reaction, and 3) the ionic composition of the various solutions is not different enough to change the rate of reaction. That the first assumption is true was shown by Holmberg and Laurell (5) and also has been demonstrated in our laboratory by the fact that the serum from patients with Wilson's disease who lack ceruloplasmin, ppd is oxidized at essentially the same rate as in saline controls. The second assumption seems validated by the findings of Holmberg and Laurell (14) and by the fact that the measured rate of oxidation almost always remains constant except in mixtures containing more than about 1 mg. of ceruloplasmin. With respect to the third assumption (20), the ionic composition of the various solutions of these experiments undoubtedly differs. Thus, Solution 2 contains about 2 mcg. of ionic copper per ml. which are not present in Solutions 3 and 4. Further, Solutions 3 and 4 have had practically all of their cations and anions exchanged, respectively, for sodium and acetate because of the columnizations to which they are subjected. However, the amount of ionic copper present in Solution 2 can be neglected since there is no significant difference between its enzymatic activity and that of solutions which lack free copper but are otherwise identical in composition to Solution 2. Furthermore, Solution 2 contains about as much acetate buffer as the columns contribute to Solutions 3 and 4, and differences in the content of other anions can probably be neglected (20). Enzymatic analyses of these solutions, carried out in this manner, appear to be useful as quantitative indicators of whether the protein has lost enzymatic activity.

Procedure. Pipette 1.0 ml. of the ceruloplasmin solution into a clean, dry 1.0-cm. path-length cuvette and add, successively, 1.5 ml. of 0.15 M sodium chloride, and 0.5 ml. of a 2.0 per cent solution of recrystallized paraphenylenediamine dihydrochloride (Fisher No. P 98) in an acetate buffer of pH 5.20 ± 0.02, and ionic strength 1.2. Make 8 to 10 timed readings at about one-minute intervals of the optical density of the solution against saline at 530 mμ. The spectrophotometer must be equipped with thermospacers to maintain the cuvette chamber at 30.0 ± 0.1° C. The rate of autoxidation of ppd, which is significant only when the unknown contains less than 0.1 mg. of ceruloplasmin per ml., can be measured using a control solution containing no ceruloplasmin. The pH of the solutions should be 5.00 ± 0.04 at the end of the determination.

Plot the optical densities obtained against time. The slope of the straight line on which the last 6 to 8 points should fall represents the rate of oxidation of ppd, and is proportional to the concentration of ceruloplasmin in the solution.

From eight sets of duplicate determinations the standard deviation of the slopes obtained was 9×10^{-4} ΔO.D. per minute.

It is possible to calibrate this procedure with known amounts of ceruloplasmin and relate the measured slope to ceruloplasmin concentration. This may be done in

[3] For convenience we have calculated ceruloplasmin concentration from ΔO.D. at 610 mμ, using the equivalent extinction coefficient to that used by Holmberg and Laurell at 605 mμ, *i.e.*, $\epsilon^{1\%}_{1\,\text{cm.}} = 0.68$, since no appreciable difference in the ΔO.D. of ceruloplasmin solutions can be detected at these two wavelengths.

TABLE II

Determination of free copper in solutions of ceruloplasmin

	Tubes		
	1	2	3
Unknown solution or blank	3.0 ml.	3.0 ml.	3.0 ml.
50% ethanol	0.4 ml.		
50% ethanol saturated with DCO		0.4 ml.	0.4 ml.
0.15 M NaCl	0.4 ml.	0.4 ml.	
Standard copper:			
1 mcg. per ml.			0.4 ml.
$O.D._{600\ m\mu}^{5\ cm.*}$ A-3	0.039	0.084	0.187
$O.D._{600\ m\mu}^{5\ cm.*}$ Blank	−0.020	−0.004	0.103

* Read one hour after addition of all reagents.

saline or in serum. The details and results of such a calibration, which are not relevant to the present experiments, will be reported subsequently.

Quantitative determination of copper—Total copper

The procedure developed in this laboratory for the quantitative determination of the total copper content of biological materials using wet digestion and dicyclohexanoneoxalyldihydrazone (DCO) (Eastman Organic Chemicals, No. 7175) is a combination and modification of two procedures described in the literature (21, 22). The method, details of which will be published in a separate paper, has been applied to ceruloplasmin solutions, serum and urine. In analyses of ceruloplasmin solutions containing from 1.4 to 3.7 mcg. of copper, the standard deviation of the results was 0.039 mcg. of copper, with recoveries of added copper of 96 to 102 per cent.

Quantitative determination of copper—Free copper

In a solution containing ceruloplasmin and free ionic copper, DCO will react only with the ionic copper to produce a deep blue color at a pH of from 7 to 9.

Procedure. Each unknown sample is diluted with water to contain no more than 0.5 mcg. of free copper per ml., and then further diluted with an equal volume of 25 per cent sodium acetate solution. Reagents are added to three 3.0-ml. aliquots of each unknown and blank solution, in the manner indicated in Table II. Optical densities are read one hour later. The optical densities given in Table II are the actual analyses of one determination on Solution A-3 (see Table IV), and the corresponding blank.

Calculations. The calculation of the free copper content of the unknown solution requires that the rise in optical density in unknown Tube 2 be corrected for the optical density of unknown Tube 1 and for the optical density due to the free copper in the reagents as measured in the blank. Tube 3 is the standard in both unknowns and blank sets. For example, the calculation of the results given in Table II is as follows:

$$\text{Free copper in A-3 (mcg. per ml.)} = \frac{0.084 - 0.039 - \left(0.016 \times \frac{0.103}{0.107}\right)}{0.103} \times 0.4 \times \tfrac{3}{5} = 0.078$$

In the numerator of this equation the first term is the optical density produced in the unknown solution by DCO, and the second term is the optical density of the same solution without DCO. The third term is the optical density due to the combination of DCO with the copper in the blank, corrected by the ratio of the optical densities produced by DCO and 0.4 mcg. of copper in

TABLE III

Components and preparative treatment of solutions 1 to 5

Solution	AK-38S*	Std. A†	Std. B‡	Solution A-1	Solution B-1	Acetate buffer pH 5.8, Γ/2 =0.1	Ascorbic acid (10 mg./ml.)	Water	Treatment
	ml.	ml.	ml.	ml.	ml.	ml.	ml.	ml.	
A-1	4.0	4.0							None
A-2				2.0		10.0			Diluted with water to 25.0 ml.
A-3				2.0		4.0	2.0		Columnized§
A-4				2.0		4.0		2.0	Columnized§
A-5		1.0				4.0	2.0	1.0	Columnized§
B-1	4.0		4.0						None
B-2					2.0	10.0			Diluted with water to 25.0 ml.
B-3					2.0	4.0	2.0		Columnized§
B-4					2.0	4.0		2.0	Columnized§
B-5			1.0			4.0	2.0	1.0	Columnized§

* See Table I for free and ceruloplasmin copper content of AK-38S.

† Std. A made to contain 44.5 micrograms of copper per ml. and 240,000 counts per minute per ml. at arbitrary zero time.

‡ Std. B made to contain 44.5 micrograms of copper per ml. and 385,000 counts per minute per ml. at arbitrary zero time. Both standards were made from quantitative mixtures of standard cupric sulfate solutions and radioactive cupric acetate.

§ Passed through resin column, of composition given in text, at 2 to 4 drops per minute. The first 21.0 ml. of effluent were rejected and the next 20.0 ml., representing all of the material introduced into the column which could be recovered, were collected, diluted to 25.0 ml. with water, and labelled A-3, A-4, A-5, *etc.*

TABLE IV

Results of experiments to demonstrate exchange of ceruloplasmin copper and free ionic copper *

Solution	Free copper	Total copper	Ceruloplasmin copper	% of Solution 2	Ceruloplasmin $\Delta O.D._{610}^{4cm.} \times 0.34$	% of Solution 2	Enzymatic activity	% of Solution 2	Net corrected counts/min. per ml.†
	mcg./ml.	*mcg./ml.*	*mcg./ml.*		*mg./ml.*		*ΔO.D./min.*		
A-2	1.98	3.74	1.76	100	0.274	100	0.0182	100	10,280
A-3	0.078	1.46	1.38	78	0.190	69	0.0153	84	2,585
A-4	0.005	1.36	1.35	77	0.247	90	0.0133	73	27
A-5									2
B-2	2.00	3.69	1.69	100	0.288	100	0.0170	100	15,017
B-3	0.045	1.41	1.36	80	0.212	74	0.0136	80	3,708
B-4	0.018	1.36	1.34	79	0.224	78	0.0131	77	31
B-5									0
Std. A	44.0§	44.3§							240,000‡

* All values given are averages of duplicate determinations except total coppers, which are averages of triplicates; ceruloplasmin coppers, which are calculated as (total-free) copper; and the free copper of B-3, which is a single determination.

† Counts are corrected for background and 12.8 hour half-life of copper[64]. Self-absorption assumed equal in all planchettes.

‡ Value given is 25 times net corrected c.p.m. measured in a 1:25 dilution of Std. A.

§ Theoretical value is 44.5 mcg. per ml.

the unknown and in the blank solutions. The factor $\frac{3}{5}$ corrects the final result for the two-fold dilution of the unknown by the 25 per cent sodium acetate and for the 3-ml. aliquot taken.

The standard deviation of the method, in 12 sets of duplicate determinations on solutions with an ionic copper concentration of 0.005 to 0.2 mcg. per ml., was 0.0115 mcg. per ml.

The accuracy of the method and the fact that it actually measures only non-ceruloplasmin copper are shown by the results in Table IV where analyses of Solutions A-2 and B-2 which, undiluted, contained 1.88 mcg. per ml. of free copper and the same amount of ceruloplasmin copper gave 1.98 and 2.00 mcg. per ml. for free copper, respectively. Table IV also shows that very low levels of free copper were found in the 3 and 4 solutions, which had been passed over ion-exchange columns to remove ionic copper, but which still contained about 1.4 mcg. per ml. of ceruloplasmin copper.

RESULTS

The composition and method of preparation of Solutions 1 to 5, for the A and B experiments, are shown in Table III. When the ascorbic acid was added in the preparation of Solution 3, the ceruloplasmin instantly turned colorless. The effluent from the column, however, was blue, indicating that the decolorization is reversible. The quantitative results of the experiments are given in Table IV.

DISCUSSION

The results will be discussed with reference to 1) whether they demonstrate that exchange of ceruloplasmin copper with ionic copper occurred, 2) the extent of such exchange, 3) the mechanism of the exchange, and 4) a hypothesis relating ceruloplasmin to absorption of dietary copper and to Wilson's disease.

1) To show that exchange of ceruloplasmin copper with free ionic copper occurred, it is necessary to find (a) a significant number of counts in the effluent solution above those accounted for by any free ionic copper, together with (b) ceruloplasmin which has not increased its copper content. It will be seen from the results given in Table IV and the following discussion that significant exchange occurred when ceruloplasmin was mixed with copper[64] in the presence of ascorbic acid.

From the results given in Table IV on Solutions A-4 and B-4, it is apparent that no exchange of ceruloplasmin copper with radioactive copper took place when the two were mixed in buffer without ascorbic acid. The 27 and 31 c.p.m. found in A-4 and B-4, respectively, are accounted for by the small amount of free copper found in these effluents since, for A-4, $\frac{0.005}{1.98} \times 10{,}280 = 26$ c.p.m. and, for B-4, $\frac{0.018}{2.00} \times 15{,}017 = 135$ c.p.m. The other control solutions demonstrated that ascorbic acid does not prevent removal of free ionic copper by the columns since Solutions A-5 and B-5 show neither free copper nor counts. Both of these sets of results also show that the columns used effec-

tively remove over 99 per cent of the free ionic copper present in A-2 and B-2. It is also clear that no appreciable amount of the copper content of the resins is released into these solutions.

In Solutions A-3 and B-3, significant exchange occurred since a) the free copper found in the effluent solutions cannot account for the counts found, and b) there is no evidence that the effluent ceruloplasmin contained a greater amount of copper than before treatment.

a) The free copper present in these solutions, though higher than that in the A-4 and B-4 solutions, accounts for only $\frac{0.078}{1.98} \times 10{,}280 = 405$ c.p.m. in A-3, and $\frac{0.045}{2.00} \times 15{,}017 = 338$ c.p.m. in B-3. Furthermore, there is only about a 2 per cent chance that either 0.078 or 0.045 mcg. of copper per ml. could be larger than 0.102 or 0.080, respectively, since 0.078 ± 0.024 and 0.045 ± 0.035 are the 99 per cent confidence ranges of each of these values based on the precision of the method and the number of samples analyzed.[4] Even the upper limits of these values for free copper could not account for as many as 20 per cent of the counts found in A-3 and B-3.

b) It is unlikely that in Solutions A-3 and B-3 radioactive copper was simply bound tightly to ceruloplasmin in addition to the copper already there. This is primarily because the amount of ceruloplasmin copper found in A-3 and B-3, 1.38 and 1.36 mcg. per ml., respectively, is almost exactly the same as that found in A-4 and B-4, 1.35 and 1.34 mcg. per ml., respectively, where no exchange occurred. These ceruloplasmin copper values indicate that about 80 per cent of the ceruloplasmin in A-2 and B-2 was recovered in the columnized 3 and 4 solutions, the rest presumably remaining in the column. Therefore, the conclusion that radioactive copper was not simply added to the ceruloplasmin in A-3 and B-3 is strengthened by the fact that the recovery of ceruloplasmin in the 3 and 4 solutions is also roughly 80 per cent when calculated on the basis of the ratio of the intensity of blue color ($\Delta O.D._{610}$) or enzymatic activity in A-3, A-4, B-3, and B-4 to these values in A-2 and B-2.

2) The results given in Table IV can be treated quantitatively to calculate the fraction of ceruloplasmin copper which was exchanged under the experimental conditions. In these calculations we shall take into account the loss of ceruloplasmin in the columns, using ceruloplasmin copper as a measure of the amount of ceruloplasmin recovered. The quantitative conclusions reached would be only slightly different if the intensity of blue color or enzymatic activity were used for estimating the recovery of ceruloplasmin.

Let m = Ceruloplasmin copper in Solution 2 in mcg. per ml.
m′ = Ceruloplasmin copper in Solution 3 in mcg. per ml.
n = Ionic copper in Solution 2 in mcg. per ml.
n′ = Ionic copper in Solution 3 in mcg. per ml.
α = Net corrected counts per minute per ml. in Solution 2.
β = Net corrected counts per minute per ml. in Solution 3.
x = Fraction of ceruloplasmin copper exchanged.

Since the counts found in Solution 3, corrected for the amount of ceruloplasmin recovered, must equal the counts incorporated into the ceruloplasmin plus the counts due to the free copper which leaked through the column,

$$\frac{m}{m'}\beta = \frac{mx\,\alpha}{n + mx} + \frac{(n')\,\frac{(m)}{(m')}\,\alpha}{n + mx}$$

and

$$x = \frac{n\beta - n'\alpha}{m'\alpha - m\beta}$$

In Solution A-3, $x = 0.448$; and in Solution B-3, $x = 0.479$. Thus, about 45 to 48 per cent of ceruloplasmin copper appears to have exchanged with the free radioactive copper in the solution under the conditions of preparation of Solution 3.

No attempt has been made, in the work described in this paper, to do more than to effect the exchange of ceruloplasmin copper with ionic copper under the one set of conditions described. In these circumstances, apparently half of the copper

[4] We are indebted to Professor John W. Fertig, of the Columbia University School of Public Health and Administrative Medicine, for his kind assistance in the statistical analysis of our results.

of ceruloplasmin, or an average of 4 atoms of copper per molecule, seems to have been removed and replaced by ionic copper from the milieu. It is obvious that the extent of the exchange reaction may be very susceptible to changes in (a) the duration of time over which the ceruloplasmin, ascorbic acid, and ionic copper are in contact, (b) the temperature at which this contact occurs, (c) the concentration of substrate or reductant added, whether ascorbic acid, as here, or some other substance, and (d) the ionic composition of the solution including, of course, the pH and the concentration of ionic copper. No attempt was made to control the first two variables and the solutions were mixed and columnized at room temperature as rapidly as was convenient. In our experiments, ascorbic acid was used instead of paraphenylenediamine, the substrate toward which ceruloplasmin is most active (14), because considerable difficulty was experienced in freeing solutions of the colored products of ppd oxidation by means of ion exchange columns. The ionic composition selected was close to that under which ceruloplasmin most rapidly catalyzes the oxidation of ppd, that is, "in acetate buffers between pH 5 and 6" (20). The ionic copper concentration was chosen to be equal to the ceruloplasmin copper concentration for simplicity.

3) Some insight into the mechanism of the exchange of ceruloplasmin copper and ionic copper may be gained by comparing our results with those obtained by Joselow and Dawson in studying the plant copper-protein, ascorbic acid oxidase (16–18). This blue-green enzyme has the same molecular weight as ceruloplasmin, 150,000, and contains 0.26 per cent of tightly bound copper which, like ceruloplasmin copper, is not removed by a sulfonic cation exchange resin. In experiments which were similar to those reported in this paper, and which were, in fact, the models for our work, these authors found that (a) there was no exchange of enzyme copper and ionic copper when the two were mixed without oxygen or substrate, (b) only when the enzyme was actively catalyzing the oxidation of ascorbic acid did exchange, to the extent of 30 per cent, occur, and (c) irreversible inactivation of the enzyme occurred apparently simultaneously with its enzymatic activity. The authors speculated that the exchange might be either the consequence of enzyme copper becoming more "accessible to . . . displacement by radiocopper" while structural rearrangements involved in the enzymatic inactivation were occurring, or that enzymatic activity was accompanied by the copper of ascorbic acid oxidase shuttling between the divalent and monovalent states, and that exchange took place when the enzyme copper was monovalent. As support for this second hypothesis, Joselow and Dawson pointed out that a shift from divalent to univalent copper may be accompanied by a change from a strong square coplanar configuration for the directed valences of copper to a weaker tetrahedral configuration. In the case of ceruloplasmin, no loss of enzymatic activity followed exchange, as is apparent from Table IV (except for the ceruloplasmin trapped in the column), so that the exchange cannot be ascribed to loosening of the copper-protein bond due to permanent structural rearrangement. Therefore, the possibility is even greater in the case of ceruloplasmin than in that of ascorbic acid oxidase that exchange is consequent to reduction of the ceruloplasmin copper to the monovalent state.

4) Since about half of the copper in ceruloplasmin can be exchanged for ionic copper, the conclusion seems inescapable that ceruloplasmin can release at least about half of its tightly bound copper into the solution reversibly. It may be of interest to speculate as to the possible relevance of such release to the relation of ceruloplasmin to absorption of dietary copper, and to Wilson's disease.

The mechanism whereby copper is absorbed is unknown but the simplest assumption concerning it would be that there is diffusion of ionic copper, or copper bound to small molecules like amino acids, from intestinal lumen into blood. Such diffusion would presumably occur only if, and when, the concentration of diffusible copper in the intestinal lumen were higher than the concentration of diffusible copper in the plasma circulating through the intestine. Since the average adult ingests 2.5 to 5 mg. of copper per day (23), and the total daily volume of gastrointestinal secretions in an adult is about 8,200 ml. per day (24), the average concentration of copper in the intestinal lumen is 0.3 to 0.6 mcg. of copper per ml. In normal subjects the plasma concentration of non-ceruloplasmin copper is less than 0.1 mcg. per ml. (25), and the concentration of cerulo-

plasmin copper is about 1.1 mcg. per ml. (3). Since only non-ceruloplasmin copper appears to be diffusible across semi-permeable membranes like the placenta, and blood-brain barrier (6, 12), probably only such copper is capable of diffusing from plasma across the membranes of the intestine into its lumen. Thus, the gradient of diffusible copper, about 0.4 mcg. per ml. in intestinal lumen to 0.1 mcg. per ml. in intestinal plasma, would favor diffusion from lumen to plasma. If, however, all or part of the ceruloplasmin copper in intestinal blood were released from the protein, thereby becoming diffusible, the concentration gradient would be reversed since there would be up to 1.1 mcg. of diffusible copper per ml. of plasma. Thus, under such conditions, the concentration of ceruloplasmin present in normal subjects would give rise to sufficient diffusible copper in intestinal plasma to prevent absorption of much of the usual dietary copper. Furthermore, if such were the normal situation, a consequence of a deficiency or absence of ceruloplasmin, which is characteristic of patients with Wilson's disease (2, 3, 8), would be increased absorption of dietary copper. In fact, just such an excessive absorption of copper, in comparison with the copper absorbed by normal subjects, seems to occur in patients with Wilson's disease (12, 26–28).

Perhaps the almost completely speculative nature of this mechanism needs further emphasis. Its relation to the present work lies in the fact that our results have demonstrated that ceruloplasmin can release at least half of its copper reversibly, *in vitro*. But we have not determined that such release can occur *in vivo*, or, if it does occur, that the protein and copper can exist dissociated for an appreciable length of time. Nor have we determined if conditions exist in the intestinal circulation that would make such a release more likely to occur there than in blood in other parts of the vascular system. For example, the ratio of the concentration of ascorbic acid to that of ceruloplasmin was about 25 times as great in Solution 3 before columnization as it is in normal plasma (1–4, 29). Although the amount of ascorbic acid used in preparing Solution 3 was about 10 times greater than that necessary to reduce the ceruloplasmin, we do not know if the concentration of ascorbic acid in intestinal plasma is sufficient to reduce and bring about exchange of ceruloplasmin copper, or whether there are other reductants or substrates present that can produce these effects.

The foregoing provides a theoretical role for ceruloplasmin in regulating the absorption of copper, and in its relation to a hypothesis of the natural history of Wilson's disease which several groups of authors have proposed (2, 10–13). The subject destined to develop this illness is presumably born with a deficiency or absence of ceruloplasmin. Absorption of a greater portion of the dietary copper than occurs in normal subjects would result from this deficiency, perhaps in the manner proposed above. After a sufficient period of time, which probably varies both with the concentration of ceruloplasmin and the amount of copper ingested, symptoms and signs of the disease appear. These are apparently due to the tissue damage which the deposits of the excess copper produce (30, 31), although the mechanism of copper toxicity is unknown. The clinical features of the disease apparently reflect injury to those organs which seem most susceptible to damage by copper, *i.e.*, the liver (32), brain and kidney (33), since the copper seems to be deposited rather ubiquitously in the body (12).

SUMMARY

About half of the 8 atoms of copper present in each molecule of the plasma protein, ceruloplasmin, have been shown to be capable of exchanging with radioactive copper^{64}. The exchange has been produced only when the protein and copper^{64} are mixed in the presence of sufficient ascorbic acid to reduce reversibly the blue ceruloplasmin to its colorless form. The existence of such an exchange implies that ceruloplasmin copper can be reversibly released from the protein, at least in the presence of ascorbic acid. It is probable that reduction of ceruloplasmin copper to the monovalent form is essential for exchange to occur.

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REFERENCES

1. Holmberg, C. G., and Laurell, C.-B., Investigations in serum copper. II. Isolation of the copper containing protein, and a description of some of its properties. Acta chem. Scandinav., 1948, **2**, 550.

2. Scheinberg, I. H., and Gitlin, D., Deficiency of ceruloplasmin in patients with hepatolenticular degeneration (Wilson's disease). Science, 1952, 116, 484.
3. Markowitz, H., Gubler, C. J., Mahoney, J. P., Cartwright, G. E., and Wintrobe, M. M., Studies on copper metabolism. XIV. Copper, ceruloplasmin and oxidase activity in sera of normal human subjects, pregnant women, and patients with infection, hepatolenticular degeneration and the nephrotic syndrome. J. Clin. Invest., 1955, 34, 1498.
4. Scheinberg, I. H., Relation of ceruloplasmin and plasma copper to hepatolenticular degeneration (Wilson's disease) *in* Progress in Neurobiology: 1. Neurochemistry, Korey, S. R., and Nurnberger, J. I., Eds. New York, Paul B. Hoeber, Inc., 1956, pp. 52–63.
5. Holmberg, C. G., and Laurell, C.-B., Oxidase reactions in human plasma caused by coeruloplasmin. Scandinav. J. Clin. & Lab. Invest., 1951, 3, 103.
6. Scheinberg, I. H., Cook, C. D., and Murphy, J. A., The concentration of copper and ceruloplasmin in maternal and infant plasma at delivery. J. Clin. Invest., 1954, 33, 963.
7. Adelstein, S. J., Coombs, T. L., and Vallee, B. L., Metalloenzymes and myocardial infarction. I. The relation between serum copper and ceruloplasmin and its catalytic activity. New England J. Med., 1956, 255, 105.
8. Bearn, A. G., and Kunkel, H. G., Abnormalities of copper metabolism in Wilson's disease and their relationship to the aminoaciduria. J. Clin. Invest., 1954, 33, 400.
9. Scheinberg, I. H., Dubin, D. T., and Harris, R. S., The survival of normal ceruloplasmin in patients with hepatolenticular degeneration (Wilson's disease). J. Clin. Invest., 1955, 34, 961.
10. Bearn, A. G., Genetic and biochemical aspects of Wilson's disease. Am. J. Med., 1953, 15, 442.
11. Earl, C. J., Moulton, M. J., and Selverstone, B., Metabolism of copper in Wilson's disease and in normal subjects. Studies with Cu-64. Am. J. Med., 1954, 17, 205.
12. Cartwright, G. E., Hodges, R. E., Gubler, C. J., Mahoney, J. P., Daum, K., Wintrobe, M. M., and Bean, W. B., Studies on copper metabolism. XIII. Hepatolenticular degeneration. J. Clin. Invest., 1954, 33, 1487.
13. Walshe, J. M., Penicillamine, a new oral therapy for Wilson's disease. Am. J. Med., 1956, 21, 487.
14. Holmberg, C. G., and Laurell, C.-B., Investigations in serum copper. III. Coeruloplasmin as an enzyme. Acta chem. Scandinav., 1951, 5, 476.
15. Bickel, H., Schultze, H. E., Grüter, W., and Göllner, I., Versuche zur Coeruloplasminsubstitution bei der hepatocerebralen Degeneration (Wilsonsche Krankheit). Klin. Wchnschr., 1956, 34, 961.
16. Joselow, M., and Dawson, C. R., The copper of ascorbic acid oxidase. Experiments with an ion exchange resin. J. Biol. Chem., 1951, 191, 1.
17. Joselow, M., and Dawson, C. R., The copper of ascorbic acid oxidase. Exchange studies with radioactive copper. J. Biol. Chem., 1951, 191, 11.
18. Dawson, C. R., The copper protein, ascorbic acid oxidase *in* Copper Metabolism. A Symposium on Animal, Plant and Soil Relationships, McElroy, W. D., and Glass, B., Eds. Baltimore, The Johns Hopkins Press, 1950, pp. 18–47.
19. Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L., Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. J. Am. Chem. Soc., 1946, 68, 459.
20. Holmberg, C. G., and Laurell, C.-B., Investigations in serum copper. IV. Effect of different anions on the enzymatic activity of coeruloplasmin. Acta chem. Scandinav., 1951, 5, 921.
21. Eden, A., and Green, H. H., Micro-determination of copper in biological material. Biochem. J., 1940, 34, 1202.
22. Peterson, R. E., and Bollier, M. D., Spectrophotometric determination of serum copper with biscyclohexanoneoxalyldihydrazone. Anal. Chem., 1955, 27, 1195.
23. Gubler, C. J., Copper metabolism in man. J. A. M. A., 1956, 161, 530.
24. Gamble, J. L., Chemical Anatomy, Physiology and Pathology of Extracellular Fluid. A Lecture Syllabus. Cambridge, Mass., Harvard University Press, 1950.
25. Gubler, C. J., Lahey, M. E., Cartwright, G. E., and Wintrobe, M. M., Studies on copper metabolism. IX. The transportation of copper in blood. J. Clin. Invest., 1953, 32, 405.
26. Zimdahl, W. T., Hyman, I., and Cook, E. D., Metabolism of copper in hepatolenticular degeneration. Neurology, 1953, 3, 569.
27. Matthews, W. B., The absorption and excretion of radiocopper in hepato-lenticular degeneration (Wilson's disease). J. Neurol., Neurosurg., & Psychiat., 1954, 17, 242.
28. Bearn, A. G., and Kunkel, H. G., Metabolic studies in Wilson's disease using Cu^{64}. J. Lab. & Clin. Med., 1955, 45, 623.
29. Hawk, P. B., Oser, B. L., and Summerson, W. H., Practical Physiological Chemistry, 13th ed. New York, The Blakiston Co., Inc., 1954, p. 1235.
30. Mallory, F. B., The relation of chronic poisoning with copper to hemochromatosis. Am. J. Path., 1925, 1, 117.
31. Mallory, F. B., and Parker, F., Jr., Experimental copper poisoning. Am. J. Path., 1931, 7, 351.
32. Franklin, E. C., and Bauman, A., Liver dysfunction in hepatolenticular degeneration. A review of eleven cases. Am. J. Med., 1953, 15, 450.
33. Bearn, A. G., Yü, T. F., Ritterband, A. B., and Gutman, A. B., Renal clearance studies in Wilson's disease. Federation Proc., 1956, 15, 12.

THE INFLUENCE OF ETHANOL UPON MAINTAINED WATER DIURESIS IN MAN

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A number of reports indicate that in normal subjects the magnitude of the diuretic response to a large intake of water varies directly with the contemporaneous rate of solute excretion by the kidney (1–6). A previous communication from this laboratory (4) described a characteristic decline in the rate of urine flow from the initial peak value which follows administration of water. This decline takes place despite maintenance of the water load. It is more regularly observed in the seated than in the recumbent subject and is associated with a concomitant decline in the excretion rate of solute. Although it was considered unlikely that antidiuretic hormone (ADH) plays a role in the decline in diuresis, this possibility has never been excluded. It was therefore decided to determine the effect of administration of ethyl alcohol, a known potent inhibitor of antidiuretic hormone release (7–10), upon the decline in diuresis. The observations described in the present report support the view that the decline in rate of urine flow does not depend on antidiuretic hormone. However, in a minority of the studies some augmentation of urine flow followed alcohol administration, pointing to a pre-existent low level of antidiuretic activity.

MATERIALS AND METHODS

The subjects were nine adult males free from renal, endocrine and cardiovascular disease. Their ages ranged from 22 to 48 years. Three were healthy physicians; the other six volunteers were patients under treatment for mild skin disorders or other minor ailments. One of the physicians (J. R.) and one of the patients (B. C.) had previously served as subjects for numerous experiments requiring a maintained water load. None of the subjects regularly drank alcohol to excess, although there was considerable variation in the customary alcoholic intake of the various members of the group. One of the patients, F. F., drank very rarely, whereas another patient, B. C., occasionally drank heavily for several successive days. No dietary restrictions were imposed except in the case of B. C., whose dietary sodium intake was limited to 25 mEq. daily at the time of his second study.

Each study was begun after breakfast when the subject voided, was weighed, and received an oral water load of 20 ml. per kilo taken over a period of 20 to 40 minutes. Unless otherwise stated, the subjects remained seated in a straight chair throughout the study, except when they stood up to void and to be weighed, at intervals of 20 or 30 minutes. After each voiding, sufficient water was drunk to restore body weight to the value observed after the initial water loading. The water diuresis was continued in this fashion until the rate of diuresis had declined by at least 1.5 ml. per minute (range 1.6 to 7.6), at which time, approximately two to six hours after the mid-point of the period of initial water loading, alcohol was given. In seven experiments on six subjects the alcohol was taken as 120 ml. of 100 proof Bourbon whiskey (48 Gm. of ethyl alcohol); one subject took 120 ml. of 86 proof Scotch whiskey (41.3 Gm. of alcohol); two subjects received intravenously 50 ml. (40 Gm.) of ethyl alcohol in a liter of a 5 per cent solution of invert sugar. The alcohol was taken over a period varying from 10 to 60 minutes, during which time, and for 50 to 200 minutes thereafter, urine collection continued and water losses were replaced as before. Samples of venous blood were obtained at the onset of water diuresis, just before alcohol was given and at the end of each experiment. Determinations were made of osmolality (expressed for convenience as milliosmols per liter), creatinine, sodium, potassium, chloride in serum and urine and of serum total protein, hemoglobin and hematocrit by methods previously employed in this laboratory (11, 12). Alcohol in blood and urine was determined by the procedure of Jetter and McLean (13) adapted for the Coleman Jr. spectrophotometer. In specimens of serum and urine containing alcohol, total solute concentration was taken as total osmolality minus alcohol concentration since the latter is freely diffusible and exists at equal concentration in blood and urine (14, 15).[1]

[1] It is recognized that solutes which undergo partial back-diffusion in the renal tubule (*e.g.*, urea) obligate water; such obligation depends on the osmotic gradient exerted by the moiety restrained from free back-diffusion. However, no effective osmotic force can be exerted by alcohol since, as pointed out by Homer Smith (16): "At equilibrium, the U/P ratio of alcohol per unit of

TABLE I

Data pertaining to subjects whose rate of urine flow increased appreciably after ethanol administration

Subject	Elapsed time	Urine flow	Urine conc.*	Renal excretion rate of				Renal clearance of			Urine alcohol conc.*
				Sodium	Potassium	Chloride	Total solute*	Creat.	Total solute* (C_{osm})	Free water (C_{H_2O})	
	mins.	*ml./min.*	*milliosm./L.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μOsm./min.*	*ml./min.*	*ml./min.*	*ml./min.*	*mM/L.*
F. F.	110–150	19.0	59	145	156	172	1,120	153	4.0	15.0	
24 yrs. 88.5 Kg.	270–330	14.2	57	119	65	105	812	142	3.0	11.2	
Lymphedema	330–370	12.5	59	94	51	78	738	140	2.7	9.8	
Seated	370–402	11.4	61	74	44	72	691	144	2.5	8.9	
	(390–453)†										
	402–470	10.9	75	100	21	74	812	156	3.0	7.9	10.0
	470–502	14.2	51	101	18	80	730	146	2.6	11.6	8.7
	502–530	14.8	48	101	25	72	716	151	2.6	12.2	6.7
P. H.	25–85	11.4	85	166	166	176	970	131	3.6	7.8	
42 yrs. 90.1 Kg.	85–145	9.2	87	113	153	131	798	121	2.9	6.3	
Low back strain	145–205	8.2	87	090	123	102	711	127	2.6	5.6	
Seated	205–265	6.1	88	060	60	059	535	127	2.0	4.1	
	265–327	9.5	87	103	31	081	815	138	3.0	6.5	9.4
	(270–325)†										
	327–385	9.4	65	079	14	059	614	133	2.3	7.1	13.6
	385–455	9.3	62	071	18	058	564	127	2.1	7.2	7.8
C. J.	74–99	14.9	59	181	89	225	880	150	3.2	11.7	
32 yrs. 79.7 Kg.	99–124	13.7	61	148	88	212	834	155	3.1	10.6	
Anxiety state	154–179	9.6	62	96	54	141	598	144	2.2	7.4	
Seated	179–211	9.8	64	122	39	146	632	152	2.3	7.5	3.8
	(190–210)‡										
	211–241	11.7	59	147	15	144	683	144	2.5	9.2	22.4
	241–301	12.6	48	139	13	139	610	153	2.2	10.4	19.8
	301–331	10.7	45	86	12	87	483	149	1.8	8.9	15.9

* Total osmolal concentrations and total solute excretion rates are exclusive of alcohol excreted.
† Fifty ml. of ethanol infused intravenously as a 5 per cent solution in 5 per cent invert sugar during this interval.
‡ One hundred and twenty ml. Bourbon whiskey (100 proof) imbibed during this interval.

Free-water clearance (C_{H_2O}) was calculated as the difference between urine flow (V) and osmolar clearance (C_{osm}) as follows:

$$C_{H_2O} = V - U_{osm}V/P_{osm},$$

in which U_{osm} and P_{osm} are osmolality of urine and plasma, respectively.

RESULTS

In Table I are presented data pertaining to the three experiments in the three subjects whose urine flow, after declining from its initial peak value, showed a distinct rise associated with the administration of alcohol. Data for consecutive periods during the course of one experiment are given in Figure 1. Urine osmolality regularly declined following ethanol administration, the greatest fall for each subject being 13, 26, and 19 milliosmols per liter. Each subject of this group manifested a small rise in endogenous creatinine clearance (range 8 to 12 ml. per min.) during the period in which alcohol was given, but urine osmolality (corrected for alcohol content) did not fall during this period, and only in one of the three subjects (P. H.) did urine flow rise significantly prior to the fall in concentration. In subject P. H. the initial increase in flow was also associated with a 50 per cent rise in total solute excretion, but the increased rate of flow was maintained despite subsequent decline in total solute excretion and creatinine clearance when urine concentration fell. Thus, in all three subjects ethanol administration was followed by increments in the rate of urine flow which could be ascribed neither to increased excretion of solute nor to increased glomerular filtration rate, as reflected by endogenous creatinine clearance. On the other hand, these increments were associated with rises in free-water clearance and reduction in urine osmolality. The changes observed in these three subjects are those that would be anticipated if release of antidiuretic hormone had been incompletely suppressed prior

water is close to 1.0. Apparently the tubules, at least the distal tubules, and the collecting ducts are freely permeable to it."

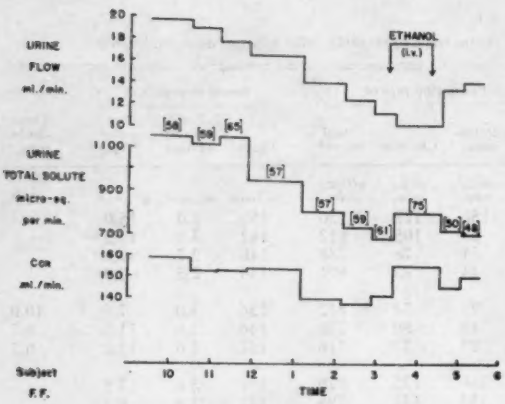

FIG. 1. AUGMENTATION OF URINE FLOW FOLLOWING ETHANOL INFUSION

Bracketed numbers above line indicating total solute excretion are values for urine osmolality.

to ethanol administration, and more complete suppression was accomplished by the alcohol.

The decline in the rate of urine flow which preceded alcohol administration was not associated with a rise in urine osmolality, but was accompanied by a decline in solute excretion and in free-water clearance.

The data in Table II pertain to the seven experiments in six subjects whose urine flow, following its initial decline, did not increase after alcohol was given. Data for consecutive periods throughout a single experiment are presented in Figure 2. In four experiments the rate of diuresis continued to fall; in three it remained essentially constant after alcohol administration. Urine osmolality (corrected for alcohol content) remained virtually unchanged after alcohol was given in three experiments, rose by four milliosmols per liter in one and fell by six to nine milliosmols per liter in three. Changes in free-water clearance after alcohol were small, ranging from a fall of 1.6 to a rise of 0.6 ml. per minute. The absence of any clear-cut increase in urine flow or reduction in osmolality after alcohol was given points to preexistent suppression of antidiuretic hormone release that was virtually complete.

DISCUSSION

Hypotonicity of the body fluids must be established in order to attain a state of "physiologic diabetes insipidus" in the normal organism. However, the presence of hypotonicity alone does not ensure that suppression of antidiuretic hormone (ADH) release will be achieved. A great many stimuli other than changes in effective osmolality influence the supra-optico-hypophyseal mechanism (11). Under the conditions of the experiments reported above, possible stimuli for ADH release include emotional factors, the pain of venapuncture, and changes in the distribution of body fluids (10, 17–24). However, in none of the experiments were the usual features of ADH effect manifest. The abrupt fall in rate of urine flow with passage of darker and more concentrated urine, which may follow a painful stimulus or other disturbing experience in the course of a water diuresis, was not observed in these studies. The lowest rate of urine flow observed was 6.1 ml. per minute, and the highest osmolality 88 milliosmols per liter. The gradual, modest fall in rate of urine flow was not associated with the increase in urinary osmolality, which would be anticipated if ADH were operative. Finally, since alcohol is a potent inhibitor of ADH release, failure to observe augmentation in the rate of flow or a decrease in the osmolality of the urine when alcohol was given in seven of ten experiments is taken to indicate that suppression of ADH activity was virtually complete. Therefore, the decline in rate of urine flow in these subjects cannot reasonably be ascribed to ADH release. The failure to observe

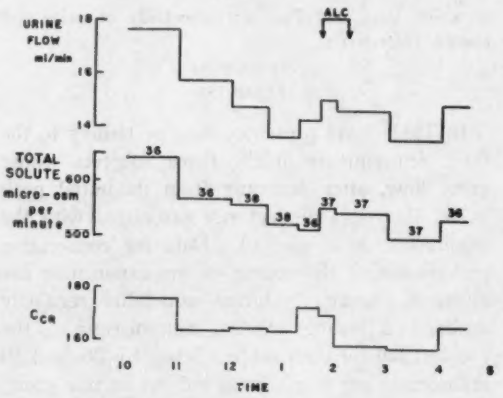

FIG. 2. FAILURE OF URINE FLOW OR CONCENTRATION TO CHANGE AFTER ALCOHOL INGESTION

Numbers above line indicating total solute excretion are values for urine osmolality. Subject B. F.

TABLE II

Data pertaining to subjects whose rate of urine flow did not rise appreciably after ethanol administration*

Subject	Elapsed time	Urine flow	Urine conc.†	Renal excretion rate of				Renal clearance of			Urine alcohol conc.†
				Sodium	Potassium	Chloride	Total solute†	Creat.	Total solute† (C_{Osm})	Free water (C_{H_2O})	
	mins.	*ml./min.*	*milli-osm./L.*	*μEq./min.*	*μEq./min.*	*μEq./min.*	*μOsm./min.*	*ml./min.*	*ml./min.*	*ml./min.*	*mM/L.*
J. R.	146–206	11.8	75	120	126	205	821	106	3.3	8.5	
42 yrs. 67.3 Kg.	236–296	7.5	79	46	36	58	595	111	2.2	5.3	
Normal	301–340‡										
Seated	357–417	7.9	70	75	18	64	561	108	2.0	5.9	20.5
B. F.	85–145	17.6	36	70	72	128	639	176	2.3	15.3	
31 yrs. 81.6 Kg.	245–305	13.9	37	54	56	91	517	167	1.9	12.0	
Normal	305–335‡										
Seated	325–385	14.6	37	74	42	92	542	158	2.0	12.6	20.8
O. D.	132–182	17.7	50	62	116	114	894	158	3.2	14.5	
22 yrs. 88.2 Kg.	292–333	16.1	50	58	95	70	802	155	2.9	13.2	
Chr. bronchitis	340–351‡										
Seated	413–453	14.9	41	29	33	18	612	162	2.2	12.7	9.7
T. T.	48–115	12.9	55	80	93	98	702	156	2.5	10.4	
32 yrs. 73.0 Kg.	115–160	11.0	56	63	67	73	618	156	2.2	8.8	
Normal	302–330§										
Seated	358–420	11.6	50	78	29	53	571	171	2.1	9.5	16.0
W. C.	80–110	19.7	38	49	90	82	750	143	2.7	17.0	
25 yrs. 67.8 Kg.	110–140‡	17.0	40	28	90	50	680	167	2.5	14.5	
Conv. polio.	145–157										
Recumbent	230–260	15.1	39	59	34	40	598	145	2.2	12.9	20.7
B. C.	140–170	10.8	74	111	102	119	802	144	3.0	7.8	
48 yrs. 67.4 Kg.	230–260	9.0	79	82	84	94	711	146	2.7	6.3	
Eczematoid derm.	302–310‡										
Recumbent	450–510	7.9	80	79	26	92	637	146	2.4	5.5	21.7
B. C.	50–80	10.3	65	47	63	42	670	134	2.5	7.8	
48 yrs. 64.8 Kg.	140–170	8.5	77	18	37	24	654	133	2.4	6.1	
Eczematoid derm.	141–157‡										
Recumbent	260–290	6.5	81	32	24	23	520	112	1.9	4.6	22.4

* Greatest rise in this group was 0.7 ml. per min. in B. F., whose urine osmolality did not change while C_{H_2O} rose slightly.

† Total osmolal concentrations and total solute excretion rates are exclusive of alcohol excreted.

‡ One hundred and twenty ml. Bourbon whiskey (100 proof) imbibed during this interval.

§ One hundred and twenty ml. Scotch whiskey (86 proof) imbibed during this interval.

a diuretic response to alcohol administration either during the initial peak of a water diuresis (10) or when, despite maintenance of the water load, urine flow declines in the seated subject indicates that a state of essentially complete physiologic diabetes insipidus may persist throughout a period of several hours at least. These observations support the view that, in the absence of important changes in glomerular filtration rate, changes in solute excretion are responsible for the variations in the diuretic response to ingested water (4).

Nevertheless the data do indicate that in three of the subjects some ADH effect was operative, albeit of insufficient magnitude to produce a low rate of urine flow or a hypertonic urine. This inference is based upon the observation that in these three subjects an increase in urine flow and a fall in urine osmolality with virtually no change in creatinine clearance or solute excretion followed the administration of alcohol. It may be noted that two of these subjects seemed quite tense during the experimental period prior to ethanol administration. However, it is doubtful if ADH was responsible for the *decline* in diuresis in these three subjects since initial rates of urine flow were not restored following alcohol administration and since urine osmolality did not rise as rate of flow fell. Indeed a relatively constant osmolality was observed during the periods prior to ethanol administration with a distinct fall in concentration

after alcohol was given. It seems probable, therefore, that in these three individuals a small, relatively constant level of ADH activity was present until alcohol inhibited further release of the hormone. Obviously other patterns of response to the experimental situation might occur, including initial complete suppression of ADH release followed by slight ADH activity coincident with the subsequent decline of diuresis.

Clinical studies suggest that in certain patients with diabetes insipidus the ability to elaborate some antidiuretic hormone is not entirely abolished, since the urine may become less copious and more concentrated after administration of an extraordinarily potent antidiuretic stimulus in the form of a large dose of nicotine (25, 26). Apart from such observations in which an attendant fall in filtration rate may complicate interpretation (27, 28), the possibility has not been explored that small amounts of hormone, insufficient to produce hypertonicity of the urine and oliguria, may be released in some patients with clinical diabetes insipidus in response to more physiologic stimuli. The observations in the three subjects of the present study who appeared to have incomplete suppression of ADH release after water loading suggest that in patients with clinical diabetes insipidus the response to ethanol administration, under conditions of moderate dehydration with hypertonicity of the body fluids, might be useful in establishing the presence or absence of very small amounts of ADH.

SUMMARY

1. The influence of alcohol administration upon the decline in rate of urine flow, which characteristically occurs when a large water load is maintained in the seated normal subject, has been studied in ten experiments on nine adult males.

2. In seven experiments on six subjects ethanol administration during the decline in diuresis failed to raise the rate of flow or to lower the osmolality of the urine (corrected for alcohol content).

3. In three subjects a distinct rise in urine flow and fall in urine osmolality followed the administration of ethanol.

4. Since alcohol is known to be a potent inhibitor of antidiuretic hormone, these inferences are drawn: 1) that virtually complete physiologic diabetes insipidus may be established and maintained by maintaining a large water load, and 2) that the decline in rate of water diuresis does not require ADH release.

5. Under similar experimental conditions of maintained water loading, certain subjects continued to release very small amounts of ADH, the presence of which may be recognized only if urine flow increases and concentration falls after ethanol is administered.

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REFERENCES

1. Brodsky, W. A., and Rapoport, S., The mechanism of polyuria of diabetes insipidus in man. The effect of osmotic loading. J. Clin. Invest., 1951, **30**, 282.
2. Wesson, L. G., Jr., and Anslow, W. P., Jr., Effect of osmotic and mercurial diuresis on simultaneous water diuresis. Am. J. Physiol., 1952, **170**, 255.
3. Ladd, M., Renal excretion of sodium and water in man as affected by prehydration, saline infusion, pitressin and thiomerin. J. Applied Physiol., 1952, **4**, 602.
4. Rosenbaum, J. D., Nelson, W. P., III, Strauss, M. B., Davis, R. K., and Rossmeisl, E. C., Variation in the diuretic response to ingested water related to the renal excretion of solutes. J. Clin. Invest., 1953, **32**, 394.
5. Welt, L. G., Young, D. T., Thorup, O. A., Jr., and Burnett, C. H., A study of renal tubular phenomena under the influence of a carbonic anhydrase inhibitor. J. Clin. Invest., 1954, **33**, 972.
6. Kleeman, C. R., Epstein, F. H., and White, C., The effect of variations in solute excretion and glomerular filtration on water diuresis. J. Clin. Invest., 1956, **35**, 749.
7. Eggleton, M. G., The diuretic action of alcohol in man. J. Physiol., 1942, **101**, 172.
8. Strauss, M. B., Rosenbaum, J. D., and Nelson, W. P., III, The effect of alcohol on the renal excretion of water and electrolyte. J. Clin. Invest., 1950, **29**, 1053.
9. van Dyke, H. B., and Ames, R. G., Alcohol diuresis. Acta endocrinol., 1951, **7**, 110.
10. Kleeman, C. R., Rubini, M. E., Lamdin, E., and Epstein, F. H., Studies on alcohol diuresis. II. The evaluation of ethyl alcohol as an inhibitor of the neurohypophysis. J. Clin. Invest., 1955, **34**, 448.
11. Strauss, M. B., Davis, R. K., Rosenbaum, J. D., and Rossmeisl, E. C., "Water diuresis" produced during recumbency by the intravenous infusion of isotonic saline solution. J. Clin. Invest., 1951, **30**, 862.

12. Birchard, W. H., Prout, T. E., Williams, T. F., and Rosenbaum, J. D., Diuretic responses to oral and intravenous water loads in patients with hepatic cirrhosis. J. Lab. & Clin. Med., 1956, **48**, 26.
13. Jetter, W. W., and McLean, R., Poisoning by the synergistic effect of phenobarbital and ethyl alcohol. An experimental study. Arch. Path., 1943, **36**, 112.
14. Jetter, W. W., A critical survey of various chemical methods for determining the alcohol content of body fluids and tissues with their physiological and medicolegal significance. Quart. J. Stud. on Alcohol., 1941, **2**, 512.
15. Rubini, M. E., Kleeman, C. R., and Lamdin, E., Studies on alcohol diuresis. I. The effect of ethyl alcohol ingestion on water, electrolyte and acid-base metabolism. J. Clin. Invest., 1955, **34**, 439.
16. Smith, H. W., The Kidney. New York, Oxford University Press, 1951, p. 281.
17. Verney, E. B., The antidiuretic hormone and the factors which determine its release. Proc. Roy. Soc., London, s.B., 1947, **135**, 25.
18. Kelsall, A. R., The inhibition of water diuresis in man by ischaemic muscle pain. J. Physiol., 1949, **109**, 150.
19. Kelsall, A. R., The urinary excretion of creatinine during inhibition of water diuresis in man by ischaemic muscle pain. J. Physiol., 1951, **112**, 54.
20. Brun, C., Knudsen, E. O. E., and Raaschou, F., Post-syncopal oliguria. Kidney function and circulatory collapse. Acta med. Scandinav., 1945, **122**, 381.
21. Brun, C., Knudsen, E. O. E., and Raaschou, F., On the cause of post-syncopal oliguria. Acta med. Scandinav., 1945, **122**, 486.
22. Brun, C., Knudsen, E. O. E., and Raaschou, F., Kidney function and circulatory collapse. Post-syncopal oliguria. J. Clin. Invest., 1946, **25**, 568.
23. Pearce, M. L., and Newman, E. J., Some postural adjustments of salt and water excretion. J. Clin. Invest., 1954, **33**, 1089.
24. Newman, E. V., Metabolic adjustments to normal and disturbed circulation in man. New England J. Med., 1954, **250**, 347.
25. Lewis, A. A. G., and Chalmers, T. M., A nicotine test for the investigation of diabetes insipidus. Clin. Sc., 1951, **10**, 137.
26. Cates, J. E., and Garrod, O., The effect of nicotine on urinary flow in diabetes insipidus. Clin. Sc., 1951, **10**, 145.
27. del Greco, F., and de Wardener, H. E., The effect on urine osmolarity of a transient reduction in glomerular filtration rate and solute output during a 'water' diuresis. J. Physiol., 1956, **131**, 307.
28. Berliner, R. W., and Davidson, D. G., Production of hypertonic urine in the absence of pituitary antidiuretic hormone. J. Clin. Invest., 1956, **35**, 690.

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STUDIES ON COPPER METABOLISM. XXIII. PORTAL (LAENNEC'S) CIRRHOSIS OF THE LIVER[1]

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Disorders in the metabolism of copper have been a constant finding in hepatolenticular degeneration, a disease which involves both brain and liver (1–4). Recently, the neurologic manifestations associated with cirrhosis of the liver have received attention (5). It is, therefore, natural to inquire if derangements of copper metabolism are present in subjects with cirrhosis of the liver and if these derangements are of any importance in the pathogenesis of the disease, particularly in relation to the development of neuropsychiatric manifestations.

This idea is in no way original. Mallory (6, 7), about 30 years ago, found the copper content of alcoholic beverages to be high and suggested that the excessive deposition of copper in the liver might be the etiologic factor in the production of pigment cirrhosis. More recently, Adams and Foley (8) have considered the possible relationship between hepatic coma and hepatolenticular degeneration and have discussed the similarities and dissimilarities between these two conditions in which both liver and brain disease are found.

There is a limited amount of information which suggests that in at least some patients with cirrhosis of the liver there is hypercupremia (1, 9–11), hypercupriuria (1) and an increased concentration of copper in the liver (12–14). Porter and Adams (15) analyzed the copper content of the putamen and liver of 11 cases of chronic liver disease dying in coma and of 11 control cases. No difference was found between the two groups in the copper content of the putamen but the liver copper was increased in two-thirds of the patients dying in hepatic coma.

In none of the studies referred to above were values presented for non-ceruloplasmin copper in serum or spinal fluid copper, or for urine copper after the administration of BAL (2,3-dimercaptopropanol). Such studies would give an estimate of the size of the fraction of copper which is loosely bound to protein. It seemed possible that this fraction of copper rather than the more tightly bound fraction might be related to the neuropsychiatric manifestations of portal cirrhosis of the liver.

The purpose of this paper is to present the results of studies of copper metabolism in patients with portal cirrhosis of the liver. Values for total serum copper, ceruloplasmin, non-ceruloplasmin copper, spinal fluid copper, urine copper, urine copper following the administration of BAL, and tissue copper are presented for control subjects and for patients with cirrhosis. Because of the suggestion that thioctic acid (6, 8-dithiooctanoic acid) may be of value in the therapy of hepatic coma (16, 17), and because of the possibility that thioctic acid, a disulfide compound, may chelate with copper, the influence of this agent on serum and urine copper was also studied.

METHODS

The patients were selected from the wards of the Salt Lake General Hospital and the Veterans Administration Hospital. All gave a history of prolonged and excessive intake of alcohol and all presented clinical as well as laboratory evidence of severe cirrhosis of the liver. A subtotal gastrectomy had been performed five years previously and a portal-caval shunt was carried out two years previously on patient K. H. All of the patients were males with the exception of I. O. Of the patients on whom an autopsy was performed, all died in hepatic coma except A. S. and P. S. These two patients died following hemorrhage from esophageal varices.

The normal subjects used for blood copper studies were healthy laboratory personnel or medical students. The "normal subjects" given BAL were hospitalized psychiatric patients without psychoses or demonstrable organic disease. The five "normal subjects" on whom autopsies were performed were all adult males who died following traumatic injuries.

Total serum copper was measured by the method of

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Gubler, Lahey, Ashenbrucker, Cartwright, and Wintrobe (18). The amount of copper in the spinal fluid was determined by the technic employed for serum. Ceruloplasmin was measured immunologically by the use of rabbit antihuman ceruloplasmin serum which had been absorbed with serum from a patient with Wilson's disease (3).

Because of the deep icterus of the serum from most of the patients with cirrhosis of the liver, the "direct-reacting" copper could not be measured by the usual technic of adding sodium diethyldithiocarbamate to serum (19). Therefore, the "direct-reacting fraction" was calculated by multiplying the concentration of ceruloplasmin (μg. per 100 ml.) by 0.32 per cent (the theoretical concentration of copper in ceruloplasmin) and subtracting the product from the total serum copper (μg. per 100 ml.). In this paper this fraction is referred to as the "non-ceruloplasmin" copper in serum. This method gives values in normal subjects which are lower than those obtained by the "direct" method. As discussed previously (3), this may be due to the fact that the antiserum used contains antibodies to proteins other than ceruloplasmin and the precipitate obtained may not be pure ceruloplasmin. This method gives only a rough approximation of the amount of "non-ceruloplasmin" copper in serum and is of value for comparative purposes only.

Tissue copper determinations were performed by digesting 2 to 4-Gm. samples of tissue in 100-ml. Kjeldahl flasks with 2 ml. concentrated sulfuric acid and 10 to 20 ml. of concentrated nitric acid until the mixture was charred. Ten ml. of nitric acid and 1.0 ml. of concentrated perchloric acid were then added and the digestion was continued until the mixture was clear. The digests were cooled, transferred quantitatively to 10-ml. volumetric flasks and made to volume with copper-free water. Blanks containing only the reagents and a standard containing the reagents plus 20 μg. of copper were carried through the same digestion procedure as the samples. One-ml. aliquots of the digests were transferred to 4.0-ml. volumetric flasks. To each was added 1.0 ml. of a saturated solution of disodium hydrogen phosphate (Na_2HPO_4) and a drop of 1.0 per cent phenolphthalein in 70 per cent ethanol. Concentrated ammonium hydroxide was then added dropwise until a definite pink color developed, after which 2 N hydrochloric acid was added until the pink color was faint but still readily discernible. Finally, 0.2 ml. of a saturated solution of "Cuprizone" [2] in 50 per cent ethanol was added, followed by copper-free water to volume (20). The contents of the flasks were then transferred to centrifuge tubes, allowed to stand 45 minutes, centrifuged, and the optical density was determined with a Beckman DU spectrophotometer in a 1.0-cm. cell and at a wave length of 600 mμ.

Urine copper determinations were performed on 20 to 40-ml. aliquots of urine. The procedure used was the same as for tissue except that a saturated solution of sodium citrate was added after the addition of sodium phosphate. Citrate was added in an amount (0.2 to 0.4 ml.) just sufficient to prevent the precipitation of hydroxides following the addition of ammonium hydroxide. The use of an excess amount of citrate was avoided since it inhibited subsequent color development.

The brains were weighed and one-half was saved for histologic studies. From the other half small portions (approximately 2 grams) of the brain stem, cerebellum, cerebral cortex and basal ganglion were dissected out and weighed. The white and gray matter of the cerebellum and cerebral cortex were usually analyzed separately. However, no difference in the concentration of copper was noted between the two substances and, therefore, the results are not reported separately. The remainder of the half brain was then homogenized in a Waring blendor for 3 to 4 minutes. Approximately three ml. of copper-free water were added per gram of brain tissue. The total amount of copper in the brain was calculated from the total weight of the brain and the concentration of copper in the homogenate.

The method used for the fractionation of brain copper was essentially that of Porter and Folch (21). Total copper was determined on a 20-ml. aliquot (Fraction H) of the brain homogenate. Triplicate 20-ml. aliquots were then centrifuged for 15 minutes at 15,000 times gravity in an angle-head centrifuge at room temperature. The supernatant solutions were transferred to 100-ml. digestion flasks. The three residues were washed with 10 ml. of water, centrifuged and the supernatant solutions were added to the digestion flasks. The combined supernatant solutions were then digested and analyzed for copper (Fraction Hs). The amount of copper in one of the three residues was determined after digestion (Hr). Fifteen ml. of water were added to each of the other two residues and, after thorough mixing, the suspensions were titrated to pH 3.8 by the addition of 0.02 N hydrochloric acid. The suspensions were then centrifuged at 2,000 times gravity for 10 minutes. The supernatant solutions were digested and analyzed for copper (Hr II). The final residues were likewise analyzed for copper (Hr III).

Free blood ammonia levels were determined by the method of Seligson and Seligson (22) as modified by Bessman and Bessman (23). The concentration of fat in the liver was measured by the method of Folch, Ascoli, Lees, Meath, and LeBaron (24).

RESULTS

Normal values

Total serum copper, ceruloplasmin, non-ceruloplasmin copper, spinal fluid copper and urine copper values in normal subjects are presented in Table I. The values for ceruloplasmin and non-ceruloplasmin copper were determined on the same 10 specimens of blood on which the total serum copper was measured (3).

[2] Biscyclohexanone oxalyldihydrazone, G. F. Smith Company, Columbus, Ohio.

TABLE I

Blood and urine copper values in normal subjects

	Total serum copper	Ceruloplasmin	Non-ceruloplasmin copper	Spinal fluid copper	Urine copper
	μg./100 ml.	mg./100 ml.	μg./100 ml.	μg./100 ml.	μg./24 hrs.
No.	10	10	10	15	20
Mean	108	34	2.5†	6.2	10.5
S.D.*	9	4	3.7	2.4	6.6
Range	92–123	27–38	0–11	3–9	0–26

* S.D. refers to standard deviation.

† This figure was obtained by dividing the sum of the individual values by ten. In the four instances where the ceruloplasmin copper exceeded the total serum copper, the value for non-ceruloplasmin copper was taken as zero.

Total serum copper

The mean value for the total serum copper for the group with cirrhosis was 141 μg. per 100 ml., as compared with the normal mean of 108 μg. ($t = 2.64$; $P = < 0.02$) (Table II). The total serum copper level was above the upper limit of normal in 9 of the 15 patients.

A significant degree of correlation was not observed between the serum copper and the bilirubin values ($r = + 0.49$; $P = > 0.05$).

Ceruloplasmin

The mean value for serum ceruloplasmin for the group was 39 mg. per 100 ml., as compared with the normal mean of 34 mg. ($t = 4.59$; $P = 0.01$). The values exceeded the upper limit of normal in 7 of the 15 patients.

Non-ceruloplasmin copper

The mean value for the serum non-ceruloplasmin copper for the group was 18 μg. per 100 ml., as compared with a mean value of 2.5 μg. for the control group ($t = 7.7$; $P = < 0.001$). The values were greater than the upper limit of normal in 9 of the 15 patients.

Spinal fluid copper

The mean value for the spinal fluid copper for the group of patients was 15 μg. per 100 ml., as compared with the normal mean of 6.2 μg. ($t = 2.98$; $P = 0.005$). The values were above the upper limit of normal in 7 of the 13 patients.

A significant degree of correlation was not observed between the spinal fluid and non-ceruloplasmin copper values ($r = + 0.39$; $P = 0.20$).

Urine copper

The excretion of copper in the urine was increased in 5 of 8 patients (Table II). The mean value for the group was 45 μg. per day, as compared with 10.5 μg. per day in the normal subjects ($t = 3.95$; $P = < 0.001$).

A significant degree of correlation was not observed between the urine and the serum non-

TABLE II

Blood and urine copper values in patients with cirrhosis of the liver

Patient	Total serum copper	Ceruloplasmin	Non-ceruloplasmin copper	Spinal fluid copper	Urine copper	Total serum bilirubin
	μg./100 ml.	mg./100 ml.	μg./100 ml.	μg./100 ml.	μg./24 hrs.	mg./100 ml.
K. R.	225	56	46	33	43	30.0
C. G.	144	38	22	18	9	2.2
E. B.	105	24	28	15	34	4.7
K. H.	72	19	11	15	12	3.2
H. L.	147	46	0			42.4
C. S.	168	47	18		15	1.6
G. B.	129	40	1	6		1.6
D. L.	126	34	17	18	40	2.0
S. L.	114	35	2	9		18.0
T. S.	123	35	11	12		3.8
J. B.	123	37	5	3		
J. G.	193	47	43	9	115	23.6
I. O.	180	50	20	42		12.5
E. K.	159	45	15	9	88	32.0
D. M.	114	26	31	6		14.4
Mean	141	39	18	15	45	13.7
S.D.	38.6	10.3	14.3	11.2	38.2	

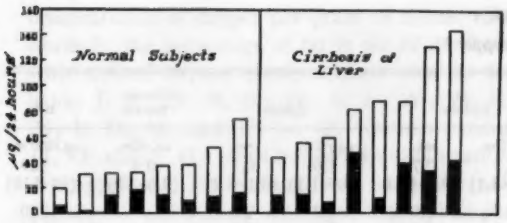

FIG. 1. THE EXCRETION OF COPPER IN THE URINE BEFORE AND AFTER THE ADMINISTRATION OF BAL

Each column represents one patient. The black portion represents the mean daily excretion of copper prior to the administration of BAL. The total column represents the excretion of copper during the 24 hours after the administration of BAL.

ceruloplasmin copper values ($r = +0.45$; $P = 0.25$).

Influence of BAL and of thioctic acid on the urinary excretion of copper

Total 24-hour urines were collected from eight normal subjects and eight patients with cirrhosis of the liver for four consecutive days. On the fourth day each subject was given 1.25 mg. BAL intramuscularly and the dose was repeated 12 hours later. The values for the three days prior to BAL were averaged for each patient. When BAL enhanced the excretion of copper, this occurred only during the day it was administered. Consequently, the post-BAL values shown in Figure 1 represent only those for the fourth day.

The normal subjects excreted an average of 12 μg. per day prior to the administration of BAL and 40 μg. on the day of BAL therapy (Figure 1). The patients with cirrhosis excreted an average of 27 μg. per day prior to BAL therapy and 87 μg. per day on the day of BAL therapy.

TABLE III

Influence of thioctic acid on the urinary excretion of copper

Patient	Thioctic acid given		Urine copper	
			Before therapy	During therapy
	mg./24 hrs.	*No. days*	*μg./24 hrs.*	
L. W.	40	1	16	22
K. H.	40	1	14	23
K. R.	40	1	89	104
K. R.	60	1	164	110
E. K.	40	3	94	194

The excretion of copper in the urine was studied in five patients following the intravenous administration of thioctic acid (Table III). In only one of the five patients (E. K.) was there a significant increase in the urinary excretion of copper.

Influence of thioctic acid on blood copper and ammonia

Three patients who were in hepatic coma were each given 20 mg. of thioctic acid intravenously (Table IV). Serum copper and blood ammonia levels were determined on blood from the right jugular vein, femoral artery and brachial vein and on spinal fluid obtained immediately prior to the administration of thioctic acid. Post-therapy values were obtained on K. H. three hours later, on I. O. four hours later, and on E. K. 5 hours later.

The administration of 20 mg. of thioctic acid had no influence on the serum or spinal fluid cop-

TABLE IV

Influence of thioctic acid on serum copper and on blood ammonia

Specimen		K. H.		I. O.		E. K.	
		Serum copper	Blood ammonia	Serum copper	Blood ammonia	Serum copper	Blood ammonia
		μg./100 ml.	*μg./100 ml.*	*μg./100 ml.*	*μg./100 ml.*	*μg./100 ml.*	*μg./100 ml.*
Jugular vein	Before	93	386	147	201	175	278
	After	93	253	159	230	160	214
Femoral artery	Before	93	371	150	180	153	326
	After	94	276	141	215	154	245
Brachial vein	Before	90	272	150	127	160	266
	After	90	227	156	138	157	243
Spinal fluid	Before	24	400	18	43		240
	After	36	316	13	75		139

TABLE V

Tissue copper *

Subject	Liver		Heart		Kidney		Spleen		Psoas muscle	Bile
	μg./Gm.	mg.	μg./Gm.	mg.	μg./Gm.	mg.	μg./Gm.	mg.	μg./Gm.	μg./100 ml.
Normals	5.3 (3.0–9.5)	8.5 (3.6–13.1)	3.2 (2.8–3.8)	1.0 (0.9–1.2)	2.2 (1.8–3.1)	0.7 (0.3–1.4)	1.0 (0.7–1.1)	0.1 (0.1–0.2)	0.9 (0.6–1.0)	329 (24–538)
H. L.	7.0	17.6	2.9	1.0	1.7	0.9	1.2	0.5		160
S. L.	3.5	5.4	3.4	1.0	1.7	0.6	0.9	0.2		171
A. S.	7.6	10.7	2.0	0.7	3.5	0.9	1.0	0.7		
J. B.	5.8	12.8	2.4	0.9	2.4	0.8	1.1	0.2		220
E. K.	3.4	12.6	2.5	1.3	3.3	0.8	1.0	0.4	0.5	350
I. O.	15.0	37.2	2.7	0.9	2.1	0.8	1.1	0.3		17
D. M.	8.0	20.1	3.6	1.3	1.6	0.7	1.1	0.1	0.8	
J. G.	29.7	41.0	2.3	0.7	2.2	1.0	1.5	0.1	0.5	2,870
W. H.	3.9	10.4	2.4	1.1	2.3	0.6	1.0	0.2	0.9	979
L. C.	4.1	8.0	2.2	0.9	1.7	0.5	0.9	0.2		
P. S.	17.1	34.2			2.6	1.5				
F. H.	8.2	18.5			1.9	0.6				
Mean	9.4	19.0	2.6	1.0	2.2	0.8	1.1	0.3	0.7	681

* The values are expressed in μg. per gram of wet tissue and in mg. in the whole organ.

per. However, in two of the patients (K. H. and E. K.) there was an appreciable decrease in the blood and spinal fluid ammonia levels. The decrease in ammonia in these two patients was associated with signs of improvement in the clinical state.

Serum copper was studied in four patients prior to the development of hepatic coma, during hepatic coma, and after the mental state had cleared and the neurologic manifestations had disappeared. A significant change in the total serum copper concentration was not observed in any of the patients.

Tissue copper

The amount of copper in the liver, heart, kidney, spleen, psoas muscle and bile was determined in 12 patients with cirrhosis of the liver (Table V).

The mean value for the concentration of copper in the liver was 9.4 μg. per Gm. of wet tissue as compared with 5.3 μg. per Gm. in the five normal subjects. The values were above the upper limit of normal in only four of the 12 patients. However, the concentration of fat in a number of the livers was increased and this would reduce the

TABLE VI

Brain copper *

Subjects	Whole brain		Brain stem	Cerebellum	Cerebral cortex	Basal ganglion	Spinal cord	
							Cervical	Lower thoracic
	μg./Gm.	mg.†	μg./Gm.	μg./Gm.	μg./Gm.	μg./Gm.	μg./Gm.	μg./Gm.
Normals	6.3 (5.1–8.3)	8.0 (7.4–10.1)	2.6 (2.0–3.0)	6.5 (4.6–7.9)	4.2 (3.9–4.7)	5.7 (4.4–6.8)	1.7 (0.3–3.0)	
H. L.	6.9	9.8	5.2	6.4	5.9			3.4
S. L.	5.1	6.0	3.6	5.4	4.2			3.0
A. S.	6.3	8.7	4.9	8.2	5.0	5.4		
J. B.	5.7	8.5	4.0	7.2	4.5	6.1		5.2
E. K.	6.3	7.6	3.5	6.7	4.6	7.4		4.1
I. O.	10.2	13.9	4.6	6.5	5.0	8.1	0.7	4.2
D. M.	7.2	10.8	6.1	8.2	7.4	10.3	1.2	
W. H.	7.7	10.4	4.5	7.4	5.9	7.3	1.7	6.2
L. C.	3.5	3.8	3.2	3.5	2.7	5.0		2.0
Mean	6.5	8.8	4.4	6.6	5.0	7.1	1.2	4.0

* The values are expressed in μg. per gram of wet tissue.
† Total mg. in the whole brain.

concentration of copper per gram of tissue. For example, the percentage of fat in six of the livers with normal copper concentrations was as follows: J. B., 29; D. M., 26; S. L., 26; W. H., 24; L. D., 18; and H. L., 12. In two patients (I. O. and J. G.) with increased concentrations of copper the fat content was increased only slightly—7 and 6 per cent, respectively. The percentage of fat in the normal livers ranged from 3 to 5.

The total amount of copper in the liver was increased above the upper limit of normal in 6 of the 12 patients with cirrhosis of the liver.

The amount of copper in the liver was greatly increased in three of the patients (J. G., I. O. and P. S.). The mean value for the group of patients with cirrhosis was 2.2 times greater than the normal mean.

The amount of copper in heart, kidneys, and psoas muscle was not increased significantly. In four of the patients there was a slight increase in the total amount of copper in the spleen. The concentration of copper in the bile varied considerably in both the "normal" subjects and the patients. Likewise, the viscosity of the bile varied considerably from sample to sample; extremely viscous, almost solid bile samples contained the highest concentration of copper.

The mean concentration and total amount of copper in the brains of nine patients with cirrhosis of the liver did not differ significantly from the normal (Table VI). In only three patients (I. O., D. M., and W. H.) was the amount of copper increased. In two of these (D. M. and W. H.) the values were only slightly above the upper limit of normal. In general, in those cases with the greatest amount of copper in the liver, the greatest amount of copper was found in the brain. The correlation coefficient between liver and brain copper was $+0.84$ ($P = < 0.01$).

The concentration of copper in the brain stem was increased in all nine of the patients; in the cerebellum in two patients; in the cerebral cortex in five patients; and in the basal ganglion in four of seven patients. However, in none of these areas was there a very great increase in the concentration of copper as compared with the normal subjects. The concentration of copper in the cervical region of the spinal cord was not increased. The concentration of copper in the lower thoracic region of the spinal cord was greater than in the cervical area but, unfortunately, at the time these studies were performed this regional difference was not appreciated and spinal cord was not obtained from the thoracic area of the "normal" subjects.

TABLE VII

Brain copper fractionation *

Subjects	Homogenate (H)	Hs	Hr	Hr II	Hr III
	μg./Gm.	%	%	%	%
Normals	6.0 (5.1–8.3)	100	58 (51–61)	42 (40–50)	9 (7–12)	34 (25–43)
H. L.	6.9	100	69	28	7	27
S. L.	5.1	100	69	30	4	25
A. S.	6.3	100	68	28	5	21
J. B.	5.7	100	72	28	6	24
E. K.	6.3	100	75	31	6	27
I. O.	10.2	100	60	38	7	33
D. M.	7.2	100	75	26	4	22
W. H.	7.7	100	72	28	5	25
L. C.	3.5	100	65	36	9	28
Mean	6.5	100	70	30	6	26

* For symbols see text.

The partition of copper in the brains of the patients with cirrhosis of the liver was significantly different from that of the "normal" subjects (Table VII). In the brains of the former, a greater portion of the copper remained in the aqueous supernatant solution after homogenization (Fraction Hs) and a greater portion of the copper remaining in the residue was released into solution after decreasing the pH to 3.8 (Fraction Hr III).

DISCUSSION

The results of this study indicate that in some patients with cirrhosis of the liver there are significant alterations in the metabolism of copper. In particular, it appears that the fraction of copper which is "loosely bound" to proteins is increased. Thus, there was a significant increase in the "non-ceruloplasmin" fraction of copper in serum, an increase in the amount of copper in urine and spinal fluid, and a greater proportion of the brain copper remained in aqueous solution after homogenization. The increased amount of copper in the urine and spinal fluid is probably a reflection of the increased amount of "non-ceruloplasmin" copper in the serum. Failure to observe a good correlation between the "non-ceruloplasmin" copper in serum and the amount

of copper in the spinal fluid and urine can probably be accounted for by the difficulty in measuring the serum fraction directly and accurately.

The possible role of estrogens in the pathogenesis of the hypercupremia associated with cirrhosis of the liver is worthy of comment. Hypercupremia may be produced in human subjects by estrogen administration (25) and is present in pregnant women (26) in whom there is a high level of circulating estrogens. In subjects with cirrhosis of the liver, there are many manifestations of excessive estrogenic stimulation because of the diminished capacity of the liver to "inactivate" the circulating estrogens (27). The observed hypercupremia may possibly be the consequence of estrogenic activity.

That the alterations in copper metabolim found in these patients account for the neuropsychiatric manifestations of this disease seems unlikely. The changes were not particularly pronounced and were not present in all patients even in the presence of severe liver disease and coma. Furthermore, the degree of alteration did not correlate with the presence or absence of coma. Thirdly, increases in "non-ceruloplasmin" copper as great or greater than those which have been observed in patients with cirrhosis have been found in pregnant women and in patients with infections in the absence of such neuropsychiatric manifestations (3). Finally, in five of the ten patients who died in hepatic coma there was no increase in total liver copper. Total brain copper was measured in eight of these patients and was found to be within the limits of normal in five. Thus, in agreement with Porter and Adams (15), we must conclude that there does not seem to be a correlation between hepatic coma and the total copper content of either the brain or the liver.

In three of the patients (I. O., D. G. and P. S.) there was a striking increase in both the concentration and total amount of copper in the liver. In these patients the total amount of copper in the liver approached the values which we have observed in Wilson's disease (49 to 124 mg.) (28). In an attempt to determine why so much more copper was present in the livers of these patients than in the livers of the other cases of cirrhosis, the clinical and pathological records were scrutinized carefully. No correlation was found between the amount of copper in the liver and the duration of liver disease or the degree or duration of jaundice. Likewise, no correlation was found between the amount of copper in the liver and the amount of fibrosis or the degree of fatty infiltration. However, in all three of the above cases with greatly elevated hepatic copper levels, in contradistinction to the other autopsied cases, there was neutrophilic infiltration in and around the small bile ducts and inspissated bile was prominent. Thus it appears that the hepatic copper level was greatly elevated in those patients with cirrhosis and associated cholangiolitis and was not increased in those without some degree of cholangiolitis. Since it has been demonstrated (4, 29, 30) that the bile is the main excretory route for copper, it is not surprising to observe that in patients with cholangiolitis there is impairment of the excretion of copper into the bile and retention of this element in the liver. To substantiate this hypothesis, the amount of copper in the liver of a young woman who died of classical primary biliary cirrhosis with xanthomatosis was determined and was found to be 426 mg. (28). These observations suggest that it would be of some interest to study copper metabolism in patients with obstructive biliary tract disease.

SUMMARY

1. Alterations in copper metabolism have been studied in a group of patients with Laennec's cirrhosis of the liver.

2. The mean value (± standard deviation) for the total serum copper was 141 ± 38.6 μg. per 100 ml.; for ceruloplasmin, 39 ± 10.3 mg. per 100 ml.; for "non-ceruloplasmin" serum copper, 18 ± 14.3 μg. per 100 ml.; for spinal fluid copper, 15 ± 11.2 μg. per 100 ml.; for urine copper, 45 ± 38.2 μg. per 24 hours. Values in normal subjects were as follows: total serum copper, 108 ±9 μg. per 100 ml.; ceruloplasmin, 34 ± 4 mg. per 100 ml.; "non-ceruloplasmin" serum copper, 2.5 ± 3.7 μg. per 100 ml.; spinal fluid copper, 6.2 ± 2.4 μg. per 100 ml.; urine copper, 10.5 ± 6.6 μg. per 24 hours.

3. Following the administration of BAL the patients excreted an average of 87 μg. of copper in the urine daily whereas the normal subjects excreted an average of 40 μg. Thioctic acid had no influence on the amount of copper excreted in the urine of the patients in either group.

4. The mean value for the total amount of copper in the liver of 12 patients with cirrhosis was 19 mg. as compared with a value of 8.5 mg. in five "normal" subjects. In six of the patients there was a normal amount of copper in the liver. In three of the patients there was a greatly increased amount of copper in this organ. The amount of copper in the heart, kidneys, muscle and spleen was not increased significantly from the normal.

5. The mean value for the total amount of copper in the brain of nine patients with cirrhosis was 6.5 mg. as compared with a value of 6.0 mg. in five "normal" subjects.

6. It is concluded from these studies that it is unlikely that the alterations in copper metabolism are of any significance in the pathogenesis of the neuropsychiatric manifestations of Laennec's cirrhosis of the liver.

7. It is suggested that in patients with cirrhosis and an associated cholangiolitis the excretion of copper into the bile is impaired and this results in retention of the element in the liver.

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REFERENCES

1. Bearn, A. G., and Kunkel, H. G., Abnormalities of copper metabolism in Wilson's disease and their relationship to the aminoaciduria. J. Clin. Invest., 1954, **33**, 400.
2. Cartwright, G. E., Hodges, R. E., Gubler, C. J., Mahoney, J. P., Daum, K., Wintrobe, M. M., and Bean, W. B., Studies on copper metabolism. XIII. Hepatolenticular degeneration. J. Clin. Invest., 1954, **33**, 1487.
3. Markowitz, H., Gubler, C. J., Mahoney, J. P., Cartwright, G. E., and Wintrobe, M. M., Studies on copper metabolism. XIV. Copper, ceruloplasmin and oxidase activity in sera of normal human subjects, pregnant women, and patients with infection, hepatolenticular degeneration, and the nephrotic syndrome. J. Clin. Invest., 1955, **34**, 1498.
4. Bush, J. A., Mahoney, J. P., Markowitz, H., Gubler, C. J., Cartwright, G. E., and Wintrobe, M. M., Studies on copper metabolism. XVI. Radioactive copper studies in normal subjects and in patients with hepatolenticular degeneration. J. Clin. Invest., 1955, **34**, 1766.
5. Summerskill, W. H. J., Davidson, E. A., Sherlock, S., and Steiner, R. E., The neuropsychiatric syndrome associated with hepatic cirrhosis and an extensive portal collateral circulation. Quart. J. Med., 1956, **25**, 245.
6. Mallory, F. B., The relation of chronic poisoning with copper to hemochromatosis. Am. J. Path., 1925, **1**, 117.
7. Mallory, F. B., Hemochromatosis and chronic poisoning with copper. Arch. Int. Med., 1926, **37**, 336.
8. Adams, R. D., and Foley, J. M., The neurological disorder associated with liver disease *in* Metabolic and Toxic Diseases of the Nervous System, Merritt, H. H., and Hare, C. C., Eds. A Research Publication, Research Nerv. & Ment. Dis. Proc., 1953, **32**, 198.
9. Heilmeyer, L., Keiderling, W., and Stüwe, G., Kupfer und Eisen als körpereigene Wirkstoffe und ihre Bedeutung beim Krankheitsgeschehen. Fischer, Jena, 1941.
10. Locke, A., Main, E. R., and Rosbash, D. O., The copper and nonhemoglobinous iron contents of the blood serum in disease. J. Clin. Invest., 1932, **11**, 527.
11. Butzengeiger, K. H., and Lange, J., "Uber das Verhalten des Eisen-und Kupferspiegels im Blut bei Erkrankungen der Leber und der Gallenwege. Deut. Arch. Klin. Med., 1952, **199**, 633.
12. Cumings, J. N., The copper and iron content of brain and liver in the normal and in hepato-lenticular degeneration. Brain, 1948, **71**, 410.
13. Schoenheiner, R., and Herkel, W., Über die Bedeutung des Kupfers für die Lebercirrhose. Klin. Wchnschr., 1930, **9**, 1449.
14. Gerlach, W., Untersuchungen über den Kupfergehalt menschlicher (und tierischer) Organe. Arch. Path. Anat., 1934, **294**, 171.
15. Porter, H., and Adams, R. D., The copper content of brain and liver in hepatic encephalopathy. J. Neuropath. & Exper. Neurol., 1956, **15**, 61.
16. Thompson, C. M., Gambescia, J. M., Lisan, P., and Fuschs, M., Addition of thioctic acid to a plan for management of hepatic insufficiency. Am. J. Med., 1956, **21**, 131.
17. Steigmann, F., and Canahuati, S. M., Thioctic acid in the treatment of hepatic coma. Federation Proc., 1956, **15**, 487.
18. Gubler, C. J., Lahey, M. E., Ashenbrucker, H., Cartwright, G. E., and Wintrobe, M. M., Studies on copper metabolism. I. A method for the determination of copper in whole blood, red blood cells, and plasma. J. Biol. Chem., 1952, **196**, 209.
19. Gubler, C. J., Lahey, M. E., Cartwright, G. E., and Wintrobe, M. M., Studies on copper metabolism. IX. The transportation of copper in blood. J. Clin. Invest., 1953, **32**, 405.
20. Peterson, R. E., and Bollier, M. E., Spectrophotometric determination of serum copper with biscyclo-

hexanoneoxalyldihydrazone. Anal. Chem., 1955, **27**, 1195.

21. Porter, H., and Folch, J., Fractionation of brain copper proteins. Federation Proc., 1954, **13**, 275.
22. Seligson, D., and Seligson, H., A microdiffusion method for the determination of nitrogen liberated as ammonia. J. Lab. & Clin. Med., 1951, **38**, 324.
23. Bessman, S. P., and Bessman, A. N., The cerebral and peripheral uptake of ammonia in liver disease with an hypothesis for the mechanism of hepatic coma. J. Clin. Invest., 1955, **34**, 622.
24. Folch, J., Ascoli, I., Lees, M., Meath, J. A., and LeBaron, F. N., Preparation of lipide extracts from brain tissue. J. Biol. Chem., 1951, **191**, 833.
25. Russ, E. M., and Raymunt, J., Influence of estrogens on total serum copper and caeruloplasmin. Proc. Soc. Exper. Biol. & Med., 1956, **92**, 465.
26. Fay, J., Cartwright, G. E., and Wintrobe, M. M., Studies on free erythrocyte protoporphyrin, serum iron, serum iron-binding capacity and plasma copper during normal pregnancy. J. Clin. Invest., 1949, **28**, 487.
27. Lloyd, C. W., and Williams, R. H., Endocrine changes associated with Laennec's cirrhosis of the liver. Am. J. Med., 1948, **4**, 315.
28. Cartwright, G. E., Gubler, C. J., and Wintrobe, M. M., Unpublished observations.
29. Van Ravesteyn, A. H., Metabolism of copper in man. Acta med. Scandinav., 1944, **118**, 163.
30. Mahoney, J. P., Bush, J. A., Gubler, C. J., Moretz, W. H., Cartwright, G. E., and Wintrobe, M. M., Studies on copper metabolism. XV. The excretion of copper by animals. J. Lab. & Clin. Med., 1955, **46**, 702.

THE DISTRIBUTION AND EXCRETION OF PHENOBARBITAL [1]

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The 5,5-disubstituted barbituric acids used medicinally are weak acids with ionization exponents in a range such that their dissociation is influenced by physiological changes of pH. There is much evidence that cellular membranes in general are permeable to the undissociated forms of weak acids and impermeable to the ionized forms (1). The experiments of Clowes, Keltch, and Krahl (2) are interpreted as indicating that a number of barbituric acid derivatives penetrate *Arbacia* eggs only as the undissociated molecules. The influence of urinary pH on the renal excretion of weak acids has been explained in terms of tubular permeability to the undissociated species and impermeability to the ionic species (3, 4). Changes in the concentration of the undissociated form of a weakly acidic drug accompanying physiological changes in the pH of blood or urine can have important effects on the distribution, excretion, and pharmacological actions of the drug.

The rate of change of the concentration of undissociated acid with pH is maximal when the pK′ of the acid equals the pH. However, of greater interest is the *proportion* by which the concentration of undissociated acid changes with a change of pH, *i.e.*, the rate at which the concentration of undissociated form is changing divided by that concentration. This function is expressed by the following equation,

$$\frac{\frac{d(\mathrm{HA})}{d(\mathrm{pH})}}{(\mathrm{HA})} = -\log_e 10 \frac{10^{\mathrm{pH}}}{10^{\mathrm{pH}} + 10^{\mathrm{pK'}}},$$

in which (HA) is the concentration of undissociated acid. In Figure 1 is shown the proportional rate of change of the concentration of the undissociated form of a weak acid with pH as a function of pK′ for the pH value of 7.4. The function is without a maximum. It is greater the lower the value of pK′.

The pK′ of phenobarbital at 37° C. and ionic strength of 0.16 (approximately the ionic strength of the inorganic ions of plasma) is 7.2. Owing to the strong electron-attracting force of the phenyl group in phenobarbital, this drug is a considerably stronger acid than the commonly used barbituric acid derivatives having only alkyl or alkenyl groups in the 5-position. The drugs of the latter types for the most part have pK′ values under the same conditions in the range of 7.6 to 8.0 (5).[3] N-methylated barbituric acid derivatives, such as hexobarbital, mephobarbital, and metharbital, have pK′ values about 0.4 unit higher than the corresponding non-methylated compounds (6). Thiobarbituric acid derivatives are stronger acids than the analogous oxygen compounds. Thiopental has a pK′ value of 7.3 at 37° C. and ionic strength of 0.16.[4] Thus phenobarbital has a lower pK′ value than any other commonly used barbituric acid derivative. It is evident from Figure 1 that the proportional change in the concentration of undissociated form resulting from a small change in pH at pH 7.4 is much greater for phenobarbital than for other hypnotic barbituric acids with pK′ values of 7.6 to 8.0. For instance, it is 1.8 times as great as for barbital or amobarbital, which have pK′ values of 7.7.

Because of the important influence that physiological shifts in pH might be expected to have on the distribution and excretion of phenobarbital, an

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[3] By spectrophotometry and by potentiometric titration we have found higher values of pK′ for several barbituric acid derivatives at 25° C. than did Krahl and also lower temperature coefficients. Our directly measured values at 37° C. and ionic strength of 0.16 are about 0.2 unit higher than the values calculated from Krahl's data.

[4] This value was determined spectrophotometrically and by potentiometric titration. The value of 8.2 reported by Brodie *et al.* (7) was obtained by titration in ethanol-water solution.

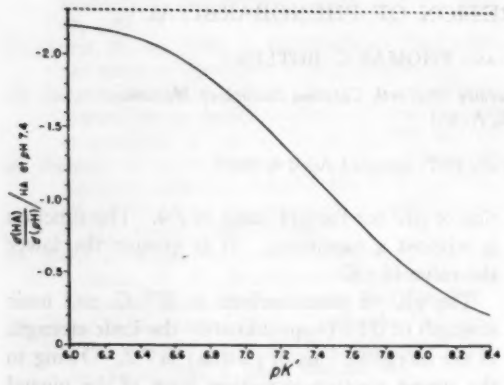

FIG. 1. THE PROPORTIONAL RATE OF CHANGE OF THE CONCENTRATION OF THE UNDISSOCIATED FORM OF A WEAK ACID WITH pH AT pH 7.4 AS IT IS RELATED TO THE pK' OF THE ACID

The dotted line is the asymptote approached by the function as pK' decreases.

investigation has been made of these effects. It was anticipated that the information to be gained might be of value in the treatment of phenobarbital poisoning. An investigation has also been made of other factors influencing the renal excretion of phenobarbital.

METHODS

Determination of phenobarbital. For dog and human plasma and for dog urine the method of Butler, Mahaffee, and Waddell (8) was used. By measurements of partition coefficients between ether and buffers, the specificity of this method for plasma had previously been demonstrated. By the same procedure it has been shown to have adequate specificity when applied to the determination of phenobarbital in dog urine. This method determines bound as well as unbound phenobarbital in plasma.

Human urine, unlike dog urine, contains unconjugated p-hydroxy phenobarbital (9), which interferes with the determination of phenobarbital. The interfering metabolic product was separated by partitioning between benzene and water. To 1 ml. of urine in a glass stoppered tube was added 1 ml. of 1 N HCl and 12 ml. of benzene. The tube was shaken and centrifuged. The aqueous phase was then transferred by pipette to a second tube, where it was shaken with a fresh 12-ml. portion of benzene. After centrifugation, the aqueous phase was removed and discarded. The first benzene extract was shaken with 1 ml. of 0.1 N HCl. After centrifugation, the aqueous phase was transferred to the second benzene extract. After shaking and centrifugation, the aqueous phase was discarded. The two portions of benzene were combined and evaporated to dryness by drawing a stream of air over the surface of the liquid with an impinger. The residue was taken up in the 1 M phosphate buffer of pH 8 used in the original method (8), and the subsequent steps of that method carried out as usual. When the specificity of this method was investigated by the procedure described by Butler, Mahaffee, and Waddell (8), the ether/buffer partition coefficients were found to conform closely to the values expected for phenobarbital.

The methods used for the determination of phenobarbital in dog and human urine when applied to urine free of drug gave calculated values that did not exceed 5 per cent of the phenobarbital concentrations that were to be experimentally measured in urine. The various drugs that were administered to dogs did not interfere with the determination of phenobarbital in plasma or urine.

Weighed samples of 1 to 5 Gm. of tissues were homogenized with weighed 5-ml. portions of 1 M phosphate buffer of pH 8. The homogenizer was a device consisting of sharp blades mounted on a shaft driven at high speed by an electric motor. A 2-ml. sample of the homogenate was weighed, 1 ml. of the buffer of pH 8 was added, and the subsequent steps of the analytical procedure were carried out in the same way as previously described for plasma (8). When this method was applied to tissues free of drug, the calculated phenobarbital concentrations did not exceed 1 μg. per Gm. Recovery of phenobarbital added to tissues was complete.

Because higher concentrations of phenobarbital were found in liver than in other tissues, the specificity of the method as applied to liver was investigated. A homogenate of a liver sample from a dog that had received phenobarbital was extracted with ether in the same manner as used in the first extraction step of the regular analytical procedure. The ether extract was evaporated and the residue distributed between ether and 1 M phosphate buffer of pH 8.2 in a 30-cell countercurrent distribution train of the type described by Craig, Hausmann, Ahrens, and Harfenist (10). Twenty-two effluent fractions of ether were withdrawn from the train. Fractions 11 to 22, which would contain p-hydroxy phenobarbital, if any were present, were analyzed for that compound by the method described by Butler (9). No evidence was found of the presence of any p-hydroxy phenobarbital. Fractions 1 to 10 were combined and evaporated and the residue was put through the countercurrent distribution train, the light solvent being a mixture of 30 per cent (v/v) ether and 70 per cent 2,2,4-trimethylpentane and the heavy solvent a 1 M phosphate buffer of pH 7.2. Thirty effluent fractions of light solvent were collected. These were individually extracted with 4-ml. portions of the buffer of pH 11 used in the regular analytical procedure. Each buffer extract was washed with 2 ml. of purified ether and the phenobarbital concentration calculated from the difference between the absorbency of the buffer at 240 mμ and that at 260 mμ. In Fractions 11 to 30 the amounts of phenobarbital measured in this way conformed quite closely to the pattern of distribution to be expected for pure phenobarbital. From the amount in the peak fraction the total phenobarbital in the tissue sample was calculated by the equation given by Bush and Densen (11). The amount so calculated was 93 per cent

of that found by the analytical procedure described in the paragraph above. Thus it has been shown that this analytical method as applied to liver has a satisfactory degree of specificity.

Measurements of binding of phenobarbital to protein. The mechanical dialyzer described by Reiner and Fenichel (12) was used. Plasma or a buffered solution of crystallized bovine serum albumin (Pentex Inc., Kankakee, Ill.) or human serum albumin (Cutter Laboratories) was placed in the dialysis bag, which was made of cellophane dialyzer tubing tied at the bottom. The outer chamber was filled with a buffer. Phenobarbital was added either to the solution in the bag alone or to the solutions on both sides of the membrane. The temperature was maintained at 37° C. and stirring of the solution in the bag was continued for 20 to 24 hours. It was found that this time was sufficient for the attainment of equilibrium. Analyses of the solutions on both sides of the membrane furnished the data for calculation of the extent of binding.

Determination of creatinine. Phillips' modification of the method of Folin and Wu (13) was used.

Administration of drugs to dogs and men. Phenobarbital was administered to dogs intravenously as a solution of the sodium salt. The men in whom clearance studies were made were receiving phenobarbital in the form of tablets by mouth. Creatinine was administered to dogs subcutaneously in aqueous solution in a dose of 100 mg. per Kg. about an hour before a clearance determination. All other drugs used in dogs were given intravenously.

Production of acidosis and alkalosis. A mixture of 29 per cent CO_2 + 71 per cent O_2 was administered to dogs with an anesthetic gas machine. A respiration pump was used to produce hyperventilation in dogs. Intravenous injections of $NaHCO_3$ in a solution of 8 Gm. per 100 ml. were given to the dogs and the men. Mice received $NaHCO_3$ intraperitoneally in a dose of 1 Gm. per Kg. as a solution of 10 Gm. per 100 ml.

Collection of urine. Urine was collected by catheter from the dogs and from one of the men in the clearance studies. The other man voided voluntarily.

Anesthetic doses in mice. Carworth Farms CF No. 1 male mice were used. In the experiments with each drug the mice were assigned to the treatments and the doses by the use of a table of random numbers. In the mice in which alkalosis was to be induced, $NaHCO_3$ was injected intraperitoneally 5 minutes before administration of the anesthetic. Phenobarbital and barbital were given intravenously as solutions of the sodium salts. A mouse was considered anesthetized if it could not gain and maintain the standing posture after stimulation by pinching of the tail. Ether was introduced in liquid form into a vessel of 1.9 L. capacity containing a mouse. The mouse was observed for 30 minutes. If at the end of that time the mouse had lost the ability to maintain the standing posture and rolled over continuously when the vessel was rotated, it was considered anesthetized. The calculated concentrations of ether were those present initially before any absorption by the mouse had taken place. Median anesthetic doses were calculated on the assumption that the curve relating log dose and proportion anesthetized is the integrated normal frequency curve.

Measurements of pH. A Cambridge Instrument Co. Model R glass electrode pH meter with water jacketed electrode assembly maintained at 37° C. was used. The primary standard of pH was a solution containing 0.025 mole of KH_2PO_4 + 0.025 mole of Na_2HPO_4 per L. It was assigned a pH value of 6.84 at 37° C. (14). Venous blood from dogs and men was used for pH measurements. Blood was taken from mice by cardiac puncture. The blood was handled with strict anaerobic precautions, and the measurements were made within 10 minutes after drawing the blood. The pH of urine samples was measured within 10 minutes after their collection.

RESULTS

Binding of phenobarbital to protein

The binding of phenobarbital to serum albumin was measured by the dialysis method. Table I shows the percentage of phenobarbital bound in a 4 Gm. per 100 ml. solution of bovine albumin as it is related to the pH of the solution. The binding is affected only to a small degree by pH over the range studied. Binding decreases as the albumin concentration decreases. In a 1 Gm. per 100 ml. solution of albumin the percentage of phenobarbital bound is about half as great as in a 4 Gm. per 100 ml. solution. In a 4 Gm. per 100 ml. solution of albumin the proportion of phenobarbital bound is almost independent of the phenobarbital concentration over the range of 20 to 100 mg. per L. (total concentrations). Binding to human serum albumin did not differ significantly from that to bovine albumin. Binding in dog and human plasma was found to be approximately the same as in a solution of pure albumin of the same concentration as the plasma albumin. Because the

TABLE I

*Binding of phenobarbital in solutions of bovine serum albumin, 4 Gm. per 100 ml. in phosphate buffers of 310 milliosmoles per L.**

pH	Per cent of phenobarbital bound
6.1	36
7.2	40
7.6	46
8.1	42
8.6	41

* The concentration of unbound phenobarbital at equilibrium was 45 mg. per L.; the temperature was 37° C.

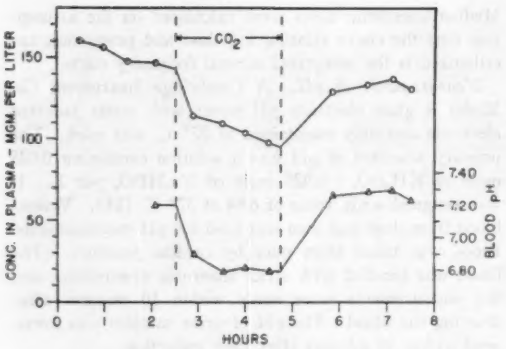

FIG. 2. EFFECTS OF INHALATION OF 29 PER CENT CO_2 IN A DOG ANESTHETIZED WITH AN INTRAVENOUS DOSE OF 125 MG. PER KG. OF PHENOBARBITAL

Phenobarbital concentrations are designated by circles, blood pH's by triangles. Times on the abscissa are measured from the time of phenobarbital administration.

extent of binding is so nearly independent of pH and of phenobarbital concentration in the ranges of interest, plasma binding was not directly measured in the experiments to be described below. In the calculations of renal clearances the assumption was made that 40 per cent of all measured plasma phenobarbital concentrations represented bound drug. This assumption is sufficiently accurate to support the conclusions derived from the calculations.

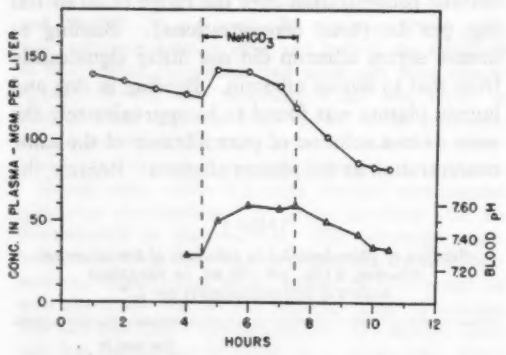

FIG. 3. EFFECTS OF INTRAVENOUS INJECTION OF $NaHCO_3$ IN A DOG ANESTHETIZED WITH AN INTRAVENOUS DOSE OF 125 MG. PER KG. OF PHENOBARBITAL

The total dose of $NaHCO_3$ administered over a period of 3 hours was 2.1 Gm. per Kg. Phenobarbital concentrations are designated by circles, blood pH's by triangles. Times on the abscissa are measured from the time of phenobarbital administration.

TABLE II

Tissue/plasma phenobarbital concentration ratios in the dog as affected by treatments changing the pH of the blood *

Treatment	Blood pH	Tissue/plasma ratio			
		Brain	Fat	Liver	Muscle
CO_2	6.76	1.2	1.0		1.0
CO_2	6.78	1.5		2.4	1.4
CO_2	6.80	1.4	1.0		1.1
CO_2	6.80	1.4	0.9	1.8	1.1
None	7.27	0.9	0.6		0.8
None	7.29	1.0	0.7		
None	7.42	0.9	0.6		0.8
Resp. pump	7.60	0.8	0.5	1.1	0.5
Resp. pump	7.70	0.7	0.6	1.1	0.6
$NaHCO_3$	7.70	0.6	0.4	1.0	0.5

* The ratios are calculated from total (bound plus unbound) concentrations in plasma. The samples were taken approximately an hour after the beginning of the treatment.

Effect of pH on the distribution of phenobarbital

In several experiments on dogs in which the blood pH was lowered by CO_2 inhalation or raised by hyperventilation or intravenous infusion of $NaHCO_3$, it was consistently observed that the plasma phenobarbital concentration fell as the blood pH fell and rose as the blood pH rose. The results of representative experiments in which acidosis was produced by CO_2 inhalation and in which alkalosis was produced by $NaHCO_3$ infusion are shown in Figures 2 and 3, respectively.

In order to investigate the changes in equilibrium between tissues and plasma that result in changes in the plasma concentration of phenobarbital, tissues were analyzed from ten dogs, three with no treatment to change pH, four made acidotic by CO_2 inhalation, and three made alkalotic by hyperventilation or $NaHCO_3$ infusion. The results are shown in Table II. In all of the tissues studied—brain, fat, liver, and muscle—the tissue/plasma concentration ratio varies in a direction opposite to that of the blood pH.

Effect of pH on anesthesia

It was noted that alkalosis produced by hyperventilation or by $NaHCO_3$ infusion caused a lightening of phenobarbital anesthesia in dogs. A quantitative assessment of this effect was made by measurement of median anesthetic doses in mice without treatment and in mice rendered alkalotic by $NaHCO_3$ administration. The dose of $NaHCO_3$ used, 1 Gm. per Kg., raised the average value

of the blood pH from the normal of 7.23 to 7.41 one-half hour after dosage. As is shown in Table III the median anesthetic dose of phenobarbital is 20 per cent higher in the mice treated with $NaHCO_3$ than in the normal. Measurement of phenobarbital in urine withdrawn by needle from the urinary bladder showed that the alkalotic mice excreted no more than about 3 per cent of the dose during the interval between the injection and the onset of anesthesia (average, about 15 minutes).

Two other anesthetics were compared with phenobarbital—barbital as an example of a drug with an ionization exponent (7.7) higher than that of phenobarbital, and ether as an example of an undissociable drug. The median anesthetic dose of barbital is raised by only 10 per cent by $NaHCO_3$ treatment. With ether there is no significant difference between the doses in mice with and without alkalosis. The probability is 0.004 that equivalent doses could differ by as much as 10 per cent.

TABLE III

Anesthetic effects of phenobarbital, barbital, and ether in normal mice and mice treated with 1 Gm. per Kg. of $NaHCO_3$ intraperitoneally *

Drug and dose	No. of mice anesthetized/total	
	Without $NaHCO_3$	With $NaHCO_3$
Phenobarbital		
146 mg./Kg.	2/10	
161	6/10	
177	9/10	1/10
195		6/10
215		10/10
Barbital		
322 mg./Kg.	5/10	1/10
354	8/10	5/10
Ether		
80 mg./L.	6/20	4/20
88	11/20	11/20
97	18/20	17/20

Calculated median doses with their standard errors

Drug	AD 50	
	Without $NaHCO_3$	With $NaHCO_3$
Phenobarbital	158± 4 mg./Kg.	191±3 mg./Kg.
Barbital	322±11 mg./Kg.	354±8 mg./Kg.
Ether	85± 2 mg./L.	87±2 mg./L.

* The doses of phenobarbital and barbital are expressed in terms of the acid forms. The values for ether are concentrations in air.

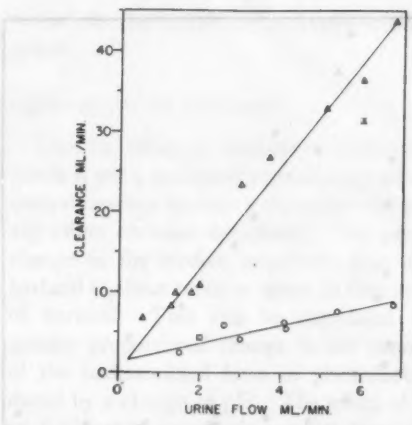

FIG. 4. RENAL CLEARANCE OF PHENOBARBITAL IN THE DOG AS IT IS RELATED TO RATE OF URINE FLOW

The values designated by circles are from experiments in which diuresis was induced by oral water, intravenous mercaptomerin, or intravenous Na_2SO_4 and in which the urinary pH was below 7.0. The values designated by triangles are from experiments in which $NaHCO_3$ was given intravenously and in which the urinary pH was 7.8 to 8.0. Clearances are calculated from the concentrations of unbound drug in plasma.

Renal excretion of phenobarbital

The renal clearance of phenobarbital was measured in dogs anesthetized with 125 mg. per Kg. of the drug. All clearances will be expressed in terms of the unbound drug in plasma (assumed to be 60 per cent of the total). Clearances based on total plasma concentrations would be 60 per cent of the values to be reported here. Under phenobarbital anesthesia without other treatment the urine flow was scanty, usually about 0.2 ml. per minute, and the phenobarbital clearance was less than 1 ml. per minute. Figure 4 shows the results of experiments in which diuresis was produced by administration of 30 ml. of water per Kg. by stomach tube, 130 mg. of mercaptomerin sodium intravenously, 100 ml. of 0.95 M Na_2SO_4 intravenously, or $NaHCO_3$ intravenously in isotonic solution or in a solution of 8 Gm. per 100 ml. and in volumes of 50 to 500 ml. Values from experiments in which diuresis was produced by water, mercaptomerin, or Na_2SO_4 are designated by circles. The pH of the urine in these experiments was less than 7.0. Values from experiments in which $NaHCO_3$ was administered are designated by triangles. In these experiments the pH of the

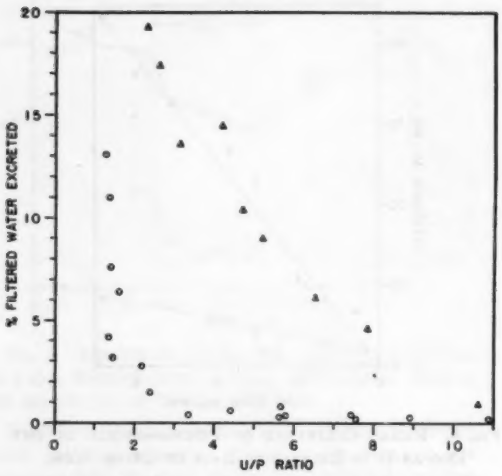

FIG. 5. THE URINE/PLASMA CONCENTRATION RATIO OF PHENOBARBITAL AS IT IS RELATED TO THE PER CENT OF FILTERED WATER EXCRETED BY THE DOG

The values designated by circles are from experiments with and without diuresis in which the urinary pH was below 7.0. The values designated by triangles are from experiments in which $NaHCO_3$ was given intravenously and in which the urinary pH was 7.8 to 8.0. The U/P ratios are calculated from the concentrations of unbound drug in plasma.

urine was 7.8 to 8.0. The clearance of phenobarbital increases with increasing urine flow in acid or alkaline urine, but at any given rate of flow the clearance is much higher in alkaline urine than in acid urine. At the same levels of urine flow and urinary pH, clearances after administration of acetazoleamide were somewhat lower than after administration of $NaHCO_3$. Probenecid did not affect the clearance of phenobarbital.

Creatinine clearances, determined simultaneously with those of phenobarbital in dogs, showed that the excretion ratio of phenobarbital was always less than unity. In acid urine it varied from 0.02 at low flows to 0.2 at high flows. In alkaline urine it varied from 0.3 at low flows to 0.7 at high flows.

Figure 5 shows the urine/plasma concentration ratio of phenobarbital plotted against the per cent of filtered water excreted, as calculated from the creatinine clearances. This chart contains some of the experiments of Figure 4 as well as some in which the dogs were not subjected to diuretic treatment. Values from experiments with or without diuretic treatment in which the urinary pH was below 7.0 are designated by circles. Values from experiments in which $NaHCO_3$ was administered and in which the urinary pH was 7.8 to 8.0 are designated by triangles. The effect of alkalinization of urine in interfering with the reabsorption of phenobarbital is again evident. With any given percentage of filtered water excreted, the U/P ratio is much higher for alkaline than for acid urine. With high flow rates the U/P ratio approaches a value somewhat over 1 for acid urine and somewhat over 2 for alkaline urine.

The renal clearance of phenobarbital was studied in two men receiving usual therapeutic doses of the drug. The results are shown in Table IV. The effects of water diuresis and of $NaHCO_3$ treatment are similar to those found in dogs. The clearance increased with increasing rate of urine flow. At comparable flows clearances were higher with alkaline than with acid urine.

DISCUSSION

Protein binding

The extent of plasma binding of phenobarbital that we have found is about the same as was measured by Lous (15, 16) in human serum by ultrafiltration. Goldbaum and Smith (17) found a higher degree of binding to albumin than we did in comparable experiments. Their calculated

TABLE IV

Phenobarbital clearances in two men receiving therapeutic doses of phenobarbital *

Man	Treatment	Urine pH	Flow	Clearance	U/P Ratio
			ml./min.	*ml./min.*	
1	Before treatment	6.7	0.8	2.5	3.1
	After water p.o.	6.6	9.0	7.5	0.9
	After water p.o.	5.5	6.7	4.3	0.7
1	Before treatment	6.1	0.8	2.0	2.6
	After $NaHCO_3$ p.o.	8.0	2.3	9.8	4.4
2	Before treatment	6.1	1.2	3.0	2.6
	Before treatment	5.8	0.8	2.0	2.4
	After $NaHCO_3$ i.v.	8.0	8.0	29.0	3.6
	After $NaHCO_3$ i.v.	8.0	2.8	13.8	4.9

* The weights of Man 1 and Man 2 were 82 and 70 Kg., respectively; the dose of water was 1 L., of oral $NaHCO_3$, 50 Gm., and of intravenous $NaHCO_3$, 14 Gm.; clearances and U/P ratios are calculated from the concentrations of unbound drug in plasma.

binding constants predict considerably more extensive binding than was found in any of our experiments. A possible explanation is that their experimental method entailed an approximate doubling of the protein concentration during the course of the ultrafiltration.

So long as the concentration of phenobarbital is low relative to the number of available binding sites on protein, the proportion bound will be nearly independent of the concentration. These conditions apparently do prevail with normal concentrations of plasma albumin and with concentrations of phenobarbital that can occur in a living animal. The small effect of pH on the extent of binding to albumin is indicative that the intrinsic binding constants for the binding of the undissociated and ionic forms of phenobarbital are nearly equal. The two forms may or may not be bound to the same sites on the protein molecule. In the physiological range of pH, binding of phenobarbital varies so little that it cannot be a factor of any importance in the changes of distribution and excretion accompanying acidosis and alkalosis.

Effect of pH on the distribution of phenobarbital

The change of plasma phenobarbital concentration resulting from change of pH is due to a change in the concentration of the ionic form. The calculated concentration of the undissociated form in plasma undergoes little change or may even change in a direction opposite to that of the total concentration. The shift of phenobarbital between tissues and plasma can be most simply explained by assuming that the intracellular pH remains relatively constant and that the cellular membrane is permeable to the undissociated form but not to the ionic form of the drug.

Brodie *et al.* (7) found that the plasma concentration of thiopental decreased during a period of acidosis. With this drug the effect is explicable in large part in terms of partitioning of the undissociated form between plasma and fat. It is surprising that alkalosis was not found to change the plasma concentration of thiopental significantly. Jailer, Zubrod, Rosenfeld, and Shannon (18) found that the plasma concentrations of quinacrine and chloroquine increased as the blood pH decreased. This effect is indicative that it is the undissociated forms of these bases that are involved in the equilibration between plasma and tissues.

Effect of pH on anesthesia

That the effect of alkalosis on barbiturate anesthesia is not a nonspecific stimulatory effect on the central nervous system is shown by the absence of any effect on ether anesthesia. The proportional change in the median anesthetic dose of phenobarbital is about twice as great as that in the dose of barbital. This can be correlated with the greater proportional change in the concentration of the undissociated form of phenobarbital produced by a change of pH. The effect of alkalosis on barbiturate anesthesia appears then to be due to the passage of some of the drug out of the brain consequent to increased ionization in the plasma.

Because of the abnormally large amount of the drug in the brain in acidosis, patients in a condition of acidosis from any cause would be expected to be unusually sensitive to the depressant effects of phenobarbital. Acidosis could be a factor in the abnormal sensitivity to phenobarbital in patients with renal disease, which was reported by Brodwall and Stöa (19), and which was attributed by them to impaired excretion of the drug.

Renal excretion of phenobarbital

Lous (15, 16) found phenobarbital clearances in man of about the same magnitude as those we have measured and observed the increase of clearance with increase of urine flow. Brodwall and Stöa (19) also reported clearances in the same range, but they did not study the relationship to urine flow. In the clearances reported by Wright (20) there is a suggestion of increasing clearances with increasing flow, but all the measurements were made at low rates of urine flow. The spectrophotometric methods used by all these workers are subject to some interference from unconjugated p-hydroxy phenobarbital in urine. In the method of Lous (15), which was also used by Brodwall and Stöa, we have found that a concentration of 100 mg. per L. of p-hydroxy phenobarbital in urine would be interpreted as a concentration of 8 mg. per L. of phenobarbital. The relative proportions of phenobarbital and unconjugated p-hydroxy phenobarbital in human urine undoubtedly vary considerably, but it is unlikely

that the clearances measured by Lous' method are in error by more than about 10 per cent owing to interference from the hydroxylated product. The method of Wright and Johns (21), which was used by Wright, may be subject to more interference than that of Lous. Cutting and Koppanyi (22) reported that the diuresis resulting from massive intravenous infusions of isotonic saline or glucose was accompanied by increased excretion of phenobarbital. The quantitative accuracy attainable with the cobalt color reaction which they used is questionable, but the effect of diuresis was so great that there can be little doubt of the qualitative validity of the observation. Only Wright (20) has attempted to study the effect of alkalinizing and acidifying treatments on phenobarbital excretion. He did not consider the effects significant, but his measurements were limited to "casual samples" of urine from one patient.

The relationship between water excretion and the urine/plasma concentration ratio as shown in Figure 5 can be interpreted, as was done by Giotti and Maynert (23) for barbital, in terms of reabsorption of phenobarbital by a process of passive back-diffusion, the distal tubule absorbing a small amount of water and being relatively impermeable to phenobarbital. The influence of the pH of the urine on excretion of phenobarbital is explicable on the assumption that the tubule is permeable only to the lipid soluble undissociated form of the drug and that equilibration between phenobarbital in the tubule and in the plasma is established either in the same region of the tubule in which the pH of the tubular fluid is changed or distal to that region. For instance if equilibrium were established between tubular fluid of pH 7.9 and plasma of pH 7.4, the concentration in the tubule would become 2.3 times as high as the unbound concentration in plasma, and the urine/plasma concentration ratio would approach this value with high rates of flow. If tubular fluid of pH 6.0 were equilibrated with the same plasma, the concentration in the tubule would be only 0.45 times the unbound plasma concentration. Thus a U/P ratio of less than unity, such as was actually observed in one of the men of Table IV, is not indicative of an active transport system but can be adequately accounted for on the assumption of passive diffusion.

Increased excretion in alkaline urine has been reported for a number of other organic acids, including salicylic acid (24–27), gentisic acid (28), and some of the sulfonamides (29). Berliner (3) has pointed out that the theory of a tubule permeable only to the undissociated form would explain the effect of pH on excretion of salicylic acid, even though with a pK′ of 3 this acid is very largely ionized in the most acid urines. The excretion of weak bases diminishes with increasing urinary pH, an effect that can also be explained in terms of tubular permeability to the undissociated species. The excretion of weak bases has been reviewed by Orloff and Berliner (4), who present a detailed derivation of the theory. This theoretical formulation is also applicable to weak acids.

It is of interest to compare our studies of the excretion of phenobarbital with those of Giotti and Maynert (23) with barbital. There are two important differences in the pattern of excretion of the two drugs. First, the clearances based on total plasma concentrations differ, that for phenobarbital being lower in acid urine and at normal flows. This is attributable to the fact that phenobarbital is extensively bound whereas barbital is not. Second, the clearance of phenobarbital is affected by the pH of the urine to a greater extent than is that of barbital. Barbital, with the higher pK′ value of 7.7, is less extensively ionized in alkaline urine than is phenobarbital. The effect of alkalinization of urine would accordingly be expected to be less for barbital.

Implications in the treatment of phenobarbital poisoning

Phenobarbital is normally the most slowly eliminated of the commonly used barbituric acid derivatives. The decrease in plasma concentration in a 24-hour period is of the order of 15 per cent in man (8, 15, 30). Because of the extreme persistence of the drug, the handling of phenobarbital poisoning entails unusual difficulties. Any measures that would increase the rate of elimination would obviously be beneficial.

There have been a number of suggestions that diuresis produced by administration of fluid or fluid together with a mercurial diuretic would be of value in treatment of poisoning with those barbituric acids that are excreted by the kidney (*e.g.*,

15, 22, 31). However, alkalinizing treatments have apparently not hitherto been advocated in phenobarbital poisoning. From the results of our experiments it would seem that the intravenous administration of $NaHCO_3$ could shorten phenobarbital coma to a very valuable degree. If the highest clearance obtained in man with intravenous $NaHCO_3$ in these experiments could be maintained, it can be calculated that the plasma concentration of phenobarbital would decline by about 40 per cent in 24 hours through the process of renal elimination alone. If metabolic inactivation accounted for an additional 5 or 10 per cent, the total elimination would be at a rate approximately three times that found normally. An additional benefit would come from the raising of the blood pH. In deep coma the depression of respiration lowers the blood pH and this causes more phenobarbital to enter the brain with further deepening of the depression. Raising the blood pH would cause the passage of some of the drug out of the brain and would lighten the depression. Thus through two different mechanisms the administration of $NaHCO_3$ might be expected to be of great value in the treatment of phenobarbital poisoning. Because of the inhibitory effect of barbituric acids on water diuresis, apprehension has been expressed that efforts to induce diuresis by administration of large volumes of fluid might lead to pulmonary edema (20). In dogs deeply anesthetized with phenobarbital it has been our experience that intravenous infusion of hypertonic solutions of $NaHCO_3$ has consistently resulted in the excretion of a larger volume of water than that administered. If the kidneys are normal, there should be no risk of fluid retention. Intravenous infusion of a concentrated solution of $NaHCO_3$ is a procedure entailing minimal risk, easily carried out without special equipment, and offering promise of great benefit in phenobarbital poisoning. It should be emphasized that this treatment could not be expected to be of nearly so much value in poisoning with any of the other familiar barbituric acids.

SUMMARY

Phenobarbital, with a pK′ of 7.2, is a stronger acid than any of the other familiar barbituric acid derivatives. The proportional change in the concentration of the undissociated form resulting from change of pH in the physiological range is greater for phenobarbital than for other barbituric acids with higher values of pK′.

In the concentrations of practical interest, phenobarbital is bound to the extent of about 40 per cent in a 4 Gm. per 100 ml. solution of serum albumin. The binding is nearly independent of pH.

In dogs the plasma phenobarbital concentration falls as the blood pH falls, and rises as the blood pH rises. These changes are due to changes in the distribution of the drug between plasma and tissues. Tissue/plasma concentration ratios vary in a direction opposite to that of the blood pH. Alkalosis, by diminishing the amount of drug in the brain, lightens phenobarbital anesthesia.

The renal clearance of phenobarbital increases with increasing rate of urine flow. At any given rate of flow, the clearance is much higher when the urine is alkaline than when it is acid. The excretion of phenobarbital can be explained on the assumptions that the drug is reabsorbed by a process of passive back-diffusion and that the renal tubule is permeable to the undissociated form and impermeable to the ionic form.

Intravenous infusion of a concentrated $NaHCO_3$ solution is suggested as a procedure of practical value in the treatment of phenobarbital poisoning.

REFERENCES

1. Jacobs, M. H., Some aspects of cell permeability to weak electrolytes. Cold Spring Harbor Symposia on Quantitative Biology, 1940, **8**, 30.
2. Clowes, G. H. A., Keltch, A. K., and Krahl, M. E., Extracellular and intracellular hydrogen ion concentration in relation to anesthetic effects of barbituric acid derivatives. J. Pharmacol. & Exper. Therap., 1940, **68**, 312.
3. Berliner, R. W., The kidney. Ann. Rev. Physiol., 1954, **16**, 269.
4. Orloff, J., and Berliner, R. W., The mechanism of the excretion of ammonia in the dog. J. Clin. Invest., 1956, **35**, 223.
5. Krahl, M. E., The effect of variation in ionic strength and temperature on the apparent dissociation constants of thirty substituted barbituric acids. J. Phys. Chem., 1940, **44**, 449.
6. Butler, T. C., The effects of N-methylation in 5,5-disubstituted derivatives of barbituric acid, hydantoin, and 2,4-oxazolidinedione. J. Am. Pharm. A. (Scient. Ed.), 1955, **44**, 367.
7. Brodie, B. B., Mark, L. C., Papper, E. M., Lief, P. A., Bernstein, E., and Rovenstine, E. A., The fate of thiopental in man and a method for its estimation in

biological material. J. Pharmacol. & Exper. Therap., 1950, **98**, 85.

8. Butler, T. C., Mahaffee, C., and Waddell, W. J., Phenobarbital: Studies of elimination, accumulation, tolerance, and dosage schedules. J. Pharmacol. & Exper. Therap., 1954, **111**, 425.
9. Butler, T. C., The metabolic hydroxylation of phenobarbital. J. Pharmacol. & Exper. Therap., 1956, **116**, 326.
10. Craig, L. C., Hausmann, W., Ahrens, E. H., Jr., and Harfenist, E. J., Automatic countercurrent distribution equipment. Anal. Chem., 1951, **23**, 1236.
11. Bush, M. T., and Densen, P. M., Systematic multiple fractional extraction procedures. Principles of application to separation of (organic) mixtures. Anal. Chem., 1948, **20**, 121.
12. Reiner, M., and Fenichel, R. L., Dialysis of protein solutions for electrophoresis. Science, 1948, **108**, 164.
13. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry, Vol. II. Baltimore, Williams & Wilkins, 1932.
14. Bates, R. G., Electrometric pH Determinations Theory and Practice. New York, Wiley, 1954.
15. Lous, P., Plasma levels and urinary excretion of three barbituric acids after oral administration to man. Acta pharmacol. et toxicol., 1954, **10**, 147.
16. Lous, P., Blood serum and cerebrospinal fluid levels and renal clearance of phenemal in treated epileptics. Acta pharmacol. et toxicol., 1954, **10**, 166.
17. Goldbaum, L. R., and Smith, P. K., The interaction of barbiturates with serum albumin and its possible relation to their disposition and pharamacological actions. J. Pharmacol. & Exper. Therap., 1954, **111**, 197.
18. Jailer, J. W., Zubrod, C. G., Rosenfeld, M., and Shannon, J. A., Effect of acidosis and anoxia on the concentration of quinacrine and chloroquine in blood. J. Pharmacol. & Exper. Therap., 1948, **92**, 345.
19. Brodwall, E., and Stöa, K. F., A study of barbiturate clearance. Acta med. Scandinav., 1956, **154**, 139.
20. Wright, J. T., The value of barbiturate estimations in the diagnosis and treatment of barbiturate intoxication. Quart. J. Med., 1955, **24**, 95.
21. Wright, J. T., and Johns, R. G. S., A study of barbiturate intoxication by an ultra-violet spectrophotometric technique. J. Clin. Path., 1953, **6**, 78.
22. Cutting, R. A., and Koppanyi, T., The effect of massive intravenous infusions on the course of barbiturate narcosis. Arch. internat. de pharmacodyn. et de thérap., 1938, **60**, 395.
23. Giotti, A., and Maynert, E. W., The renal clearance of barbital and the mechanism of its reabsorption. J. Pharmacol. & Exper. Therap., 1951, **101**, 296.
24. Smith, P. K., Certain aspects of the pharmacology of the salicylates. Pharmacol. Rev., 1949, **1**, 353.
25. Dalgaard-Mikkelsen, S., On the renal excretion of salicylate. Acta pharmacol. et toxicol., 1951, **7**, 243.
26. Gutman, A. B., Yü, T. F., and Sirota, J. H., A study, by simultaneous clearance techniques, of salicylate excretion in man. Effect of alkalinization of the urine by bicarbonate administration; effect of probenecid. J. Clin. Invest., 1955, **34**, 711.
27. Macpherson, C. R., Milne, M. D., and Evans, B. M., The excretion of salicylate. Brit. J. Pharmacol., 1955, **10**, 484.
28. Batterman, R. C., and Sommer, E. M., Fate of gentisic acid in man as influenced by alkalinization and acidification. Proc. Soc. Exper. Biol. & Med., 1953, **82**, 376.
29. Beyer, K. H., Peters, L., Patch, E. A., and Russo, H. F., The renal elimination of sulfamerazine, sulfamethazine, sulfadiazine and sulfathiazole by the dog. J. Pharmacol. & Exper. Therap., 1944, **82**, 239.
30. Lous, P., Barbituric acid concentration in serum from patients with severe acute poisoning. Acta pharmacol. et toxicol., 1954, **10**, 261.
31. Ohlsson, W. T. L., Blodsköljningsbehandling vid svår akut barbiturförgiftning. Nord. med., 1949, **42**, 1471.

APPARENT FREE AMINO ACIDS IN DEPROTEINIZED PLASMA OF NORMAL AND UREMIC PERSONS [1]

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Not all clinical manifestations of severe uremia can be explained on the basis of abnormalities of the water balance or of electrolyte patterns. Many patients die with the uremic syndrome even as the plasma levels of all known electrolytes are normal (1, 2). Even though it appears unlikely that retention of the known end products of protein metabolism is responsible for the clinical picture of uremia (3), it is probable that the toxicity of the uremic syndrome is related to a disturbance of protein metabolism. Measurement and comparison of free amino acid concentrations in the body fluids of uremic persons with the amino acid patterns in normal body fluids is prerequisite to further investigations of protein metabolism in uremia. If one or more of the essential amino acids were found deficient in uremic body fluids such a finding might serve to explain certain clinical features of the uremic syndrome which suggest decreased protein synthesis. Abnormally high concentrations of individual amino acids in uremic body fluids would raise the question of their contribution to the uremic toxicity. Consistently abnormal amino acid patterns in uremia would call for explorations of the mechanisms underlying this abnormality. Such investigations might fill in and define our presently so vacuous and chimerical picture of the derangements of chemical and physiological processes which are the basis for the uremic syndrome.

Animal experiments from our laboratories (4) have demonstrated deficiencies of apparent free methionine and threonine, with simultaneous excess of apparent free arginine and glycine in the blood plasma of nephrectomized uremic dogs. The present report extends our studies of plasma amino acid levels in uremia to human patients.

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METHODS

Plasma was obtained from six human patients with severe uremia caused by chronic glomerular nephritis. The procurement of the plasma specimens coincided with replacement transfusions which had become indicated in these patients because of severe signs and symptoms of uremia and because of the coexisting anemia. Since the blood specimens were withdrawn from the patients simultaneously with the administration of identical volumes of citrated blood, they became diluted with donor blood during withdrawal. It is, however, estimated that none of the uremic specimens contain more than 25 per cent normal donor blood because only the first 1000 ml. of the withdrawn blood were used for amino acid assay. The dilution factor would tend to diminish any differences which exist between uremic blood and bank blood, and the abnormalities actually existing in uremic blood must be more pronounced than our data would indicate. Aside from the replacement transfusions, the patients had the therapeutic regimen customary in uremic patients: water and electrolyte balance, a high-caloric, low-protein diet, and vitamin supplements. Three patients were digitalized, three received daily injections of testosterone propionate, one (E. H., No. 4) had had a course of antibiotics one week before the procurement of the plasma specimen (antibiotics may interfere with microbiological amino acid assay). Plasma samples were taken only from patients with chronic uremia due to primary renal disease because it was felt that this would reduce the variability of blood amino acid levels which might be introduced by the coexistence of uremia with debridement and digestion of necrotic tissues (as in acute renal failure or in simultaneous severe traumatic injuries).

Five hundred ml. blood samples were also taken from nine healthy young men who had been without food for ten hours.

All blood was taken from the venous side of the circulation and collected in siliconed glass bottles which contained 30 mg. heparin per 500 ml. blood. The heparinized blood was transferred to the cold room where it was allowed to settle by gravity. One or two days after collection the supernatant plasma was syphoned off, collected in siliconed glass bottles and frozen. It was preserved in the frozen state up to the time of further processing.

Deproteinization was effected by adding per ml. of plasma 1.5 ml. of approximtaely 4 per cent sodium tung-

state in 0.35 N H_2SO_4. The mixture was allowed to stand for 30 minutes with occasional shaking, the suspension was filtered on a Buchner funnel, the residue was washed twice with a minimum of distilled water, and the washings were added to the filtrate. The filtrate was evaporated to dryness under reduced pressure to residual material which was taken up in warm distilled water and, after filtration, aliquots were diluted with distilled water to a volume such that 1 ml. was equivalent to approximately 0.5 and 1.5 ml. of the original plasma, respectively.

For purposes of hydrolysis of amino acid conjugates an aliquot of the condensed filtrate of one sample was made 6 N in acid with HCl, refluxed for 24 hours, filtered, the residue washed with a minimum of warm water, and the washings added to the filtrate. This filtrate was then treated in the same manner as that described above.

The assay procedures have been described previously (5, 6).

RESULTS AND DISCUSSION

Concentration of apparent free amino acids in normal human plasma

It is evident from Table I that the authors' values for apparent free amino acids in normal plasma are comparable to values obtained elsewhere both microbiologically and by column chromatography for aspartic acid, glutamic acid, methionine, phenlalanine, serine and valine. Our values for arginine, histidine and proline were lower than concentrations observed elsewhere. Values obtained here for glycine and threonine were higher than those determined with chromatography elsewhere, and our measurements of the concentrations of isoleucine, leucine and lysine were lower

TABLE I

Concentration of apparent free amino acids in normal human plasma

Method	Microbiological assay						Column chromatography	
Series	Authors*				Hier and Bergeim (7)†	Harper *et al.* (11)‡	Stein and Moore (8)§	Huisman (12)‖
Treatment	Unhydrolyzed		Hydrolyzed		Unhydrolyzed	Unhydrolyzed	Unhydrolyzed	Unhydrolyzed
	μg./ml.	*MDM* (%)	μg./ml.	*MDM* (%)	μg./ml.	μg./ml.	μg./ml.	μg./ml.
Arginine	4.7¶ (4.2–5.6)**	3.9 (2.5–6.1)	3.1 (2.9–3.3)	3.4 (2.0–4.7)	23 (14–37)	23 (12–30)	15 (12–19)	14 (8.5–20)
Aspartic acid	2.9 (2.3–3.3)	3.5 (3.2–4.0)	6.8 (6.3–7.4)	3.2 (1.9–4.6)		3.3 (0–12)	0.3 (0.1–0.7)	2.0 (1.5–2.5)
Glutamic acid	12 (11–12)	3.2 (1.3–6.4)	20 (18–22)	4.0 (1.4–6.6)		8.9 (0–13)	7.0 (4.3–12)	8.8 (7.0–12)
Glycine	22 (18–27)	11 (6.4–18)	40 (31–50)	8.7 (1.4–16)		29 (8.0–54)	15 (13–17)	16 (14–18)
Histidine	7.0 (6.4–7.9)	6.9 (1.6–16)	7.8 (7.7–7.9)	3.0 (1.6–4.5)	14 (11–21)	21 (10–38)	12 (7.9–15)	27 (24–29)
Isoleucine	7.2 (7.1–7.2)	7.0 (6.0–8.1)	8.0 (7.9–8.0)	4.8 (1.4–8.3)	17 (12–23)	20 (12–42)	20 (6.9–13)	7.1 (4.2–10)
Leucine	16 (15–17)	2.6 (0.69–4.5)	16 (15–17)	2.4 (1.8–3.1)	20 (16–28)	25 (10–52)	17 (14–23)	16 (7.8–24)
Lysine	15 (12–17)	4.2 (2.9–6.2)	15 (12–17)	13 (1.8–24)	30 (20–37)	37 (23–58)	27 (25–30)	14 (12–17)
Methionine	4.1 (3.5–4.8)	3.4 (1.2–5.0)	3.4 (3.4–3.5)	4.8 (1.9–7.6)		5.7 (2.5–10)	3.8 (3.3–4.3)	4.2 (2.0–6.4)
Phenylalanine	11 (9.9–12)	3.8 (2.4–5.8)	12 (11–12)	3.2 (1.6–4.7)	14 (9.0–21)	20 (11–40)	8.4 (6.9–9.5)	8.6 (6.6–10)
Proline	6.6 (5.7–7.9)	1.6 (1.4–2.0)	6.4 (6.2–6.6)	1.4 (1.1–1.8)		26 (15–57)	24 (18–33)	15 (13–18)
Serine	13 (6.2–18)	11 (6.0–14)	10 (8.7–11)	4.1 (1.9–6.3)		14 (3.0–20)	11 (10–12)	
Threonine	19 (15–22)	5.8 (1.2–11)	15 (14–15)	8.4 (7.7–9.1)	21 (13–32)	21 (9.0–36)	14 (12–17)	9.8 (7.5–11)
Valine	25 (23–26)	2.1 (0.64–4.5)	26 (26–26)	1.6 (1.1–2.2)	29 (25–37)	32 (25–42)	29 (24–37)	25 (20–30)

* Pooled sample from nine normal men, fasted. Average of 2 to 3 assays at 2 to 3 different sample concentrations (0.45 to 1.5 relative to fresh plasma).
† Average value from 34 normal men, fasted.
‡ Average value from 17 normal men, fasted.
§ Average value from five normal men, fasted.
‖ Average value from two normal men, three hours after feeding.
¶ Average value.
** Range of values.

TABLE II

Concentration of apparent free amino acids in uremic human plasma from one patient (G.) and its relationship to that found in normal human plasma

Amino acid	Concentration in plasma*				Ratio of concentrations (Uremic/Normal)	
	Unhydrolyzed		Hydrolyzed		Unhydrolyzed	Hydrolyzed
	μg./ml.	MDM (%)	μg./ml.	MDM (%)		
Arginine	18† (16–21)‡	4.1 (1.1–9.4)	15 (14–16)	5.0 (4.8–5.3)	3.8† (3.6–3.8)§	4.8† (4.8–4.9)§
Aspartic acid	0.54 (0.20–0.87)	11 (8.7–13)	18 (17–19)	2.8 (2.1–3.4)	0.16 (0.06–0.26)	2.6 (2.6–2.7)
Glutamic acid	9.0 (7.9–9.5)	5.7 (1.5–9.5)	107 (91–123)	11 (5.6–17)	0.76 (0.72–0.79)	5.4 (4.1–6.8)
Glycine	52 (47–57)	2.3 (1.5–3.0)	74 (73–74)	4.3 (1.4–7.3)	2.5 (2.1–2.9)	2.0 (1.5–2.4)
Histidine	5.5 (4.9–6.1)	4.6 (3.7–8.5)	6.8 (6.2–7.4)	2.8 (0.81–4.9)	0.77 (0.77–0.78)	0.88 (0.81–0.94)
Isoleucine	3.1 (1.8–4.2)	20 (14–29)	3.0 (1.9–4.0)	13 (13–13)	0.39 (0.25–0.55)	0.38 (0.24–0.51)
Leucine	5.6 (5.1–6.2)	3.3 (2.2–5.0)	6.7 (6.1–7.3)	2.2 (1.8–2.7)	0.36 (0.32–0.41)	0.43 (0.36–0.49)
Lysine	9.6 (7.0–12)	10 (1.3–28)	15 (12–18)	8.8 (0.63–17)	0.64 (0.58–0.75)	1.0 (1.0–1.1)
Methionine	2.2 (1.8–2.6)	4.2 (3.3–5.4)	3.2 (2.8–3.7)	7.4 (3.7–11)	0.52 (0.51–0.54)	0.96 (0.82–1.1)
Phenylalanine	4.8 (4.5–5.0)	4.5 (2.0–8.0)	5.3 (5.0–5.6)	3.7 (2.8–4.6)	0.44 (0.42–0.45)	0.46 (0.45–0.47)
Proline	18 (17–18)	2.3 (1.2–3.1)	22 (21–22)	1.0 (0.59–1.5)	2.8 (2.1–3.2)	3.4 (3.2–3.6)
Serine	8.8 (8.1–9.6)	10 (7.3–13)	13 (13–13)	8.9 (4.8–13)	0.81 (0.48–1.3)	1.3 (1.2–1.5)
Threonine	8.7 (7.2–10)	6.0 (0.66–11)	11 (9.8–12)	8.4 (5.7–11)	0.46 (0.40–0.50)	0.75 (0.70–0.80)
Valine	10 (9.1–12)	2.7 (1.4–3.8)	10 (10–11)	3.2 (0.97–5.4)	0.42 (0.35–0.48)	0.40 (0.39–0.42)

* Pooled samples from a single uremic patient.
† Average values from 2 to 3 assays at 2 to 3 different sample concentrations (0.40 to 1.5 relative to fresh plasma).
‡ Range of values.
§ Range of values of ratios at different but equal plasma sample concentrations.

than data obtained elsewhere with microbiological methods.

It should be noted that there was no change in the concentration of histidine, lysine, phenylalanine, proline and valine before and after complete hydrolysis of the plasma. The mean deviation from the means observed at twelve levels of sample ranging from 0.08 to 1.2 ml. of plasma per ml. of assay sample is low (average, 4.0 per cent; range, 1.6 to 7.0 per cent). This indicated that the values found for these amino acids may represent approximately the concentrations of the free amino acids present in the plasma.

The average mean deviations for arginine (3.9 per cent), methionine (3.4 per cent), and threonine (5.8 per cent) were low, but their observed concentrations decreased after hydrolysis. This may indicate the presence in unhydrolyzed plasma of a stimulatory substance which could stimulate growth of the test organisms. Comparable decreases of apparent free arginine and serine after hydrolysis have been observed in the livers of tumorous rats (5). The high mean deviations and/or the changes following hydrolysis observed for glycine, isoleucine and serine might be ascribed to the presence of conjugated amino acids or other types of substances stimulatory or inhibitory to the growth of the assay organisms. It is assumed that average values for amino acids calculated from assay data of this character have relative significance when the conditions are rigidly standardized (4). The reliability of the observed concentrations of aspartic and glutamic acids may be questioned since spontaneous (or other) hydrolysis of asparagine and glutamine may contribute to these values (4). It is of interest that Stein and Moore (8) found no change in concentration of arginine and serine and a slight increase for threonine after hydrolysis, while the relative changes for the remaining eleven amino acids were in agreement with the present results.

Concentration of apparent free amino acids in uremic human plasma and its relation to that found in normal human plasma

It is apparent from the data in Tables II and III that histidine, isoleucine, leucine, methionine, phenylalanine, threonine and valine were significantly decreased in the plasma of Patient No. 1, and that methionine and threonine were decreased in the plasma of all six patients. The decrease of the above amino acids was apparent both in the hydrolyzed and unhydrolyzed state. It is not clear at this time whether the consistent decrease of some amino acids in uremic human plasma reflects a physiological change brought about by the uremic state as such, or whether these low amino acid levels in uremia were secondary to the prolonged administration of the low protein diet which is standard treatment in uremic patients. The overnight fasting of the donors of normal plasma specimens probably did not greatly influence the blood amino acid concentrations of these healthy young men who may have been accustomed to a high protein diet, which may explain the higher levels of many amino acids in their blood. In future experiments of this character it may become necessary to match the patients and donors as to age, hormone and vitamin level, and to induce the donors to submit to the standard uremic dietary regime for a lengthy period prior to the procurement of the plasma specimens. Irrespective of its mechanism, the reduced plasma level of the amino acids mentioned here may well contribute to the poor wound healing, diminished antibody formation and decreased hemoglobin synthesis which suggest inhibition of protein synthesis in the uremic syndrome.

It is apparent from the data in Tables II and III that the concentration of arginine, glycine and proline were markedly higher in uremic plasma than in normal plasma both before and after hydrolysis. The significant increase in apparent arginine has already been noted by Schmidt, McElvain, and Bowen (9) (who concluded, however, that such changes fell within the normal variability of their patients) and also by Dunn, Murphy, and Salisbury (4) and by Doolan, Harper, Watten, Hutchin, and Canada (10) who concluded that the increased concentration of arginine in uremic plasma might well be due to the accumulation of urea, which would retard the enzymatic conversion of arginine into urea and ornithine on the basis of a mass action. Intravenously administered arginine is not very toxic (13) but a chronically elevated arginine concentration may predispose to or cause secondary derangements of protein metabolism which are not observable in acute amino acid infusions. A toxic effect of the chronically elevated plasma arginine level in uremia is therefore not ruled out on the basis of present knowledge.

The data presented here, as well as previous measurements of plasma amino acids in uremic dogs (4), demonstrate the marked elevation of apparent free plasma glycine which occurs in the

TABLE III

Concentration of some protein metabolites in unhydrolyzed human plasma *

Substance	Normal (Pooled, ♂)	Uremic					
		G. (♂)	M. (♂)	K. (♂)	E. H.† (♂)	C. (♂)	S. G. (♀)
Creatine (mg. %)		17	18	20		16	17
Urea N (mg. %)		160	134	130	300	200	140
Arginine (μg./ml.)	4.7‡	18	12	14	3.6	13	5.4
	3.9	*4.1*	*8.3*	*3.8*	*15*	*4.4*	*6.2*
Glycine (μg./ml.)	22	52	70	122	80	32	102
	11	*2.3*	*6.4*	*10*	*32*	*4.0*	*13*
Methionine (μg./ml.)	4.1	2.2	3.6	3.8	2.0	3.0	1.7
	3.4	*4.2*	*12*	*11*	*7.6*	*10*	*10*
Proline (μg./ml.)	6.6	18	18	49	9.2	24	19
	1.6	*2.3*	*2.5*	*1.8*	*11*	*3.5*	*4.2*
Threonine (μg./ml.)	19	8.7	8.7	10	9.6	9.8	6.5
	5.8	*6.0*	*5.9*	*12*	*8.1*	*9.3*	*12*

* Data in micrograms per ml.; figures in italics are mean deviations from the mean in per cent.
† Patient E. H. received penicillin and streptomycin four days before procurement of blood specimen.
‡ Amino acid data for normal blood taken from Table II.

uremic syndrome. De Vries and Alexander (14) had already demonstrated slight elevations of free plasma glycine in five of ten patients with mild uremia and Braun, Kisfaludy, and Dubsky (15) have reported similar findings in two patients. Glycine is known to cause potassium extrusion when added to media containing suspended cells (16), and to cause severe toxic effects when administered intravenously to dogs (17) and humans (18). An investigation of the role of the glycine molecule in uremia appears indicated. It is not believed that a toxic effect of elevated plasma glycine levels in uremia is due to the increase in free plasma ammonia which has been observed following glycine infusions in dogs and patients (17). The plasma ammonia of uremic patients is not consistently elevated (19, 20), and the symptomatology of uremia cannot be reproduced by infusion of ammonia and is not identical with other clinical conditions in which plasma ammonia is increased (21). The elevated plasma glycine content of uremic plasma could be caused by a deficient glycine excretion on the part of the failing kidney, by reduced glycine transformations, or by increased glycine production in the uremic state.

The data presented here also show a distinct elevation of the proline content of uremic plasma. The sources of the excessive plasma proline and its possible contribution to the uremic symptomatology are unknown and await investigation.

In Patient No. 1 the concentration of aspartic acid, glutamic acid, methionine and lysine became markedly increased after hydrolysis while the concentration of other amino acids varied to a lesser degree. It may be inferred from these results that the concentration of asparagine and glutamine is substantially higher in uremic than in normal plasma, an inference which is consistent with the role of these substances as ammonia acceptors. The minor but significant post-hydrolysis variations in the concentration of histidine, lysine, methionine, serine and threonine suggest the existence of microbiologically inactive conjugates of these amino acids in uremic but not in normal deproteinized plasma.

While marked differences existed between the concentrations of individual amino acids in normal and uremic unhydrolyzed plasma filtrates, the net increase of total free amino acids in uremic plasma was only 93 per cent. This would seem to be in agreement with the observation that, although the urea content might increase as much as 3000 per cent, total free amino acid nitrogen in uremic plasma does not increase more than about 100 per cent (22, 23). The major portion of the large net difference following hydrolysis can be ascribed to increases of arginine, asparagine, glutamine and proline in uremic plasma. Levenson, Howard, and Rosen (23) noted the presence of a heterogeneous amino acid conjugate which tended to parallel the urea concentration in patients with severe battle wounds and mild uremia who had to be subjected to heroic and multiple therapeutic maneuvers. The principal constituents of this conjugate were usually glutamic acid, glycine, leucine and proline, whereas in normal plasma it was composed of glutamic acid, glycine and threonine.

SUMMARY

Apparent free plasma amino acids were measured with microbiological methods on a pooled plasma sample of nine normal fasting young men and on individual plasma samples of six patients with severe uremia which was secondary to chronic glomerulonephritis.

The authors' data for the apparent free amino acid levels of normal human plasma are, on the whole, comparable with data for free amino acid content of human plasma reported by other investigators.

In extension of parallel investigations of the free amino acid content of normal and uremic (nephrectomized) dog plasma, it was found that the content of free arginine, glycine and proline was definitely elevated in five of six uremic human plasma specimens. Several other amino acids appear to be significantly decreased in uremic human plasma.

REFERENCES

1. Bradley, S. E., The Pathologic Physiology of Uremia in Chronic Bright's Disease. Springfield, Thomas, 1948.
2. Merrill, J. P., The Treatment of Renal Failure. New York, Grune and Stratton, 1955.
3. Olsen, N. S., and Bassett, J. W., Blood levels of urea nitrogen, phenol, guanidine and creatinine in uremia. Am. J. Med., 1951, 10, 52.
4. Dunn, M. S., Murphy, E. A., and Salisbury, P. F., Free and apparent amino acids in deproteinized

plasma of normal and of uremic (nephrectomized) dogs. Proc. Soc. Exper. Biol. & Med., 1956, **92**, 682.

5. Dunn, M. S., and Murphy, E. A., Effect of ethionine alone or with amino acids on tumor growth, carcass weight and nonprotein amino acids in rat liver. Cancer Research, 1955, **15**, 760.
6. Dunn, M. S., Camien, M. N., Malin, R. B., Murphy, E. A., and Reiner, P. J., Percentages of twelve amino acids in blood, carcass, heart, kidney, liver, muscle, and skin of eight animals. Calif. Univ. Pub. Physiol., 1949, **8**, 293.
7. Hier, S. W., and Bergeim, O., The microbiological determination of certain free amino acids in human and dog plasma. J. Biol. Chem., 1946, **163**, 129.
8. Stein, W. H., and Moore, S., The free amino acids of human blood plasma. J. Biol. Chem., 1954, **211**, 915.
9. Schmidt, E. G., McElvain, N. F., and Bowen, J. J., Plasma amino acids and ether-soluble phenols in uremia. Am. J. Clin. Path., 1950, **20**, 253.
10. Doolan, P. D., Harper, H. A., Watten, R. H., Hutchin, M. E., and Canada, R. O., Changes in plasma amino acids and blood oxygen content during dialysis with the Kolff-Merrill artificial kidney. Trans. Am. Soc. Artificial Internal Organs, 1956, **2**, 113.
11. Harper, J. B., Hutchin, M. E., and Kimmel, J. R., Concentration of nineteen amino acids in plasma and urine of fasting normal males. Proc. Soc. Exper. Biol. & Med., 1953, **80**, 768.
12. Huisman, T. H., The concentration of different amino acids in the blood plasma in children suffering from rickets and scurvy. Pediatrics, 1954, **14**, 245.
13. Najarian, J. S., and Harper, H. A., Comparative effect of arginine and monosodium glutamate on blood ammonia. Proc. Soc. Exper. Biol. & Med., 1956, **92**, 560.
14. De Vries, A., and Alexander, R., Studies on amino acid metabolism. II. Blood glycine and total amino acids in various pathological conditions, with observations on the effects of intravenously administered glycine. J. Clin. Invest., 1948, **27**, 655.
15. Braun, P., Kisfaludy, S., and Dubsky, M., Quantitative analysis of free amino acids in normal and pathological human serum and urine. Acta med. acad. sc. Hung., 1955, **7**, 147.
16. Christensen, H. N., and Riggs, T. R., Concentrative uptake of amino acids by the Ehrlich mouse ascites carcinoma cell. J. Biol. Chem., 1952, **194**, 57.
17. Harper, H. A., Najarian, J. S., and Silen, W., Effect of intravenously administered amino acids on blood ammonia. Proc. Soc. Exper. Biol. & Med., 1956, **92**, 558.
18. Doolan, P. D., Harper, H. A., Hutchin, M. E., and Alpen, E. L., The renal tubular response to amino acid loading. J. Clin. Invest., 1956, **35**, 888.
19. Russell, D. S., Ammonia content of the blood in nephritis. Biochem. J., 1923, **17**, 72.
20. Van Slyke, D. D., Linder, G. C., Hiller, A., Leiter, L., and McIntosh, J. F., The excretion of ammonia and titratable acid in nephritis. J. Clin. Invest., 1926, **2**, 255.
21. McDermott, W. V., Jr., and Adams, R. D., Episodic stupor associated with an Eck fistula in the human with particular reference to the metabolism of ammonia. J. Clin. Invest., 1954, **33**, 1.
22. Berglund, H., Nitrogen retention in chronic interstitial nephritis and its significance. J. A. M. A., 1922, **79**, 1375.
23. Levenson, S. M., Howard, J. M., and Rosen, M. A., Studies of the plasma amino acids and amino conjugates in patients with severe battle wounds. Surg., Gynec. & Obst., 1955, **101**, 35.

FURTHER OBSERVATIONS ON THE HEMODYNAMIC EFFECT OF PLASMA VOLUME EXPANSION BY DEXTRAN [1]

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An earlier study (1) indicated that the administration of 500 ml. of 6 per cent dextran to five patients produced hemodilution and increased the cardiac output. There was insufficient evidence in this study to correlate the increase in cardiac output with any phase of the hemodynamic change resulting from plasma volume expansion. In order to extend the previous observations, right auricular, pulmonary arterial, pulmonary wedge pressure, cardiac output, and plasma volume were measured in 16 patients with varying degrees of hypervolemia following dextran injection. Data to be presented indicate that factors other than elevated right auricular, pulmonary arterial or pulmonary wedge pressures were operative in altering the response of the cardiac output of normal individuals to plasma volume expansion by a diluent such as 6 per cent dextran and by 12 per cent salt-free dextran. Increase in cardiac output was generally observed following hypervolemia.

MATERIALS AND METHODS

The patients were studied in the fasting state. All except one (No. 15) were male, and their ages ranged from 22 to 44 years. All of the 16 patients were afebrile and ambulatory. Diagnoses are listed in Table I. The partially hydrolyzed 6 per cent dextran solution had a viscosity ranging from 2.7 to 3.3 centistokes at 25° C. and was made up in 0.85 per cent saline.[2] The 12 per cent dextran solution was salt-free and had a viscosity of 8.8 centistokes at 25° C.

Intravenous catheterizations were performed with a 6-F or 8-F intracardiac catheter. The systemic arterial blood samples and pressure records were obtained from an indwelling, femoral arterial needle. An electromanometer (Sanborn) was used for all pressure tracings. Arbitrary zero reference was 5 cm. below the level of the sternal *angle of Louis*. Mean pressures were obtained by electrical integration of the pulse contours. Pulmonary wedge pressures were obtained as described by Hellems, Haynes, and Dexter (2). Peripheral venous pressure and some atrial pressures were measured with a saline manometer, using the same zero reference point as above.

Cardiac outputs were derived by the method of Kinsman, Moore, and Hamilton (3, 4) using Evans Blue Dye (T-1824). As shown in Table I, three patients (Cases 10, 11, and 12) showed variations of less than 5 per cent in consecutive dye output determinations made prior to alterations induced by plasma volume expansion. Mean pulmonary circulation time was obtained by plotting the semi-logarithmic dye curves arithmetically and determining planimetrically the mean division of the area beneath the curve. Pulmonary vascular resistance was calculated according to the formula mentioned by Dexter, Dow, Haynes, Whittenberger, Ferris, Goodale, and Hellems (5).

Blood volume was determined according to the method of Gibson and Evans (6); multiple specimens were obtained starting 10 minutes following the injection of Evans Blue Dye.

The experiments were done as follows: The patients were given mild sedation (0.1 to 0.2 gram of sodium phenobarbital intramuscularly) prior to catheterization. The catheter tip was passed either into the superior vena cava (Case 6), the right atrium, the right ventricle, or the pulmonary artery, and the indwelling femoral arterial needle was inserted. An initial dye injection into the heart was made, and several pressure determinations were made. One thousand or 1,500 ml. of 6 per cent dextran or the 12 per cent dextran were given intravenously at the rate of 25 ml. per minute. Pressure readings were recorded during the control period, midway in the administration of the dextran, immediately after its completion, and at frequent intervals thereafter. Second dye injections were done within 10 minutes after dextran administration in most patients. Other dye injections were made after 15 minutes to 2 hours. Subsequent pressure and cardiac output measurements were performed at the times recorded in Table I. Pressure readings were obtained immediately after the cardiac output.

"Plasma volume expansion" is used in the text of this paper to indicate the degree of hemodilution resulting from dextran injection, and the "initial plasma volume" is based on the apparent volume of distribution of T-1824.

RESULTS

Pertinent data are presented in Table I. Cardiac output increased in all but 3 of the 16 pa-

[1] Dextran and intrinsic viscosity measurements were kindly supplied by Dr. Homer Stavely, Commercial Solvents Corporation, Terre Haute, Indiana.

[2] Reviewed by the Veterans Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans Administration.

TABLE I

Patient	Diagnosis	Time post dextran	Cardiac output	Cardiac index	Right atrial mean pressure	Pulmonary artery pressure Systolic/ Diastolic (Mean)	Pulmonary wedge pressure	Femoral artery pressure Systolic/ Diastolic (Mean)	Peripheral venous pressure	Hematocrit
		min.	*L./min.*	*L./min./ M.²*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mm. Hg*	*mm. Hg*	%
1	Vascular headache	Basal	5.5	3.8	1				6	46
		8	7.8	3.9	6					38
		22	7.0	3.5	2					38
2	Amyotropic lateral sclerosis	Basal	6.3	3.2	2			110/80	8	47
		2	9.0	4.6	8					39
		80	7.6	3.9	6					39
3	Epigastric hernia	Basal	5.4	3.1	1			120/80	6	30
		7	7.9	4.6	9					23
		21	7.6	4.5	5					24
4	Anxiety neuroses	Basal	5.2	2.8				110/72	8	41
		16	6.8	3.4	7				8	33
		48	7.5	4.0	4				7	32
		120	5.9	3.2	4					36
5	Post-peritonsillar abscess	Basal	7.8	4.4	2			106/64	8	43
		9	9.8	5.5	7					36
		22	9.5	5.3	3					36
		45	7.6	4.3	2					38
6	Non-bleeding duodenal ulcer	Basal	6.5	4.0	4			120/80	8	47
		15	7.4	4.6	10					36
		40	7.8	4.8	10					
7	Bulbous emphysema	Basal	6.1	3.5		16/4 (9)	2	126/72 (86)		49
		9	9.4	5.3		42/9 (27)		130/72 (90)		40
		22	9.5	5.4		38/13 (24)	13	132/70 (90)		40
8	Pyoderma, left foot	Basal	7.3	4.3		23/7 (9)	4	155/80 (102)		43.5
		9	7.4	4.4		41/18 (28)	20	190/95 (128)		35.5
		25	7.7	4.5		32/9 (16)	10	175/83 (114)		36
		60	7.5	4.4		30/12 (18)	10			37
9	Eczematoid and stasis dermatitis	Basal	5.3	3.0	4	22/6 (12)	6	144/70 (95)		42
		12	7.1	4.0	8	37/14 (24)		162/78 (106)		36.5
		28	7.0	4.0	7	36/14 (24)	18	162/78 (106)		36.5
		73	7.2	4.0	6	32/6 (16)		167/78 (106)		37
10	Mucous colitis	Basal	7.0	4.1		20/6 (11)	6	120/65 (85)		43.5
		Basal	7.2	4.2		20/6 (11)	6	120/65 (85)	7	40.5
		18	8.3	4.8		30/14 (21)	14	135/70 (95)	14	33.5
		44	8.0	4.6		32/13 (21)	13	153/82 (105)	3	34
11	Previous amputation	Basal	6.1	3.0	2	22/6 (11)	5	106/52 (67)		43.5
		Basal	5.8	2.9		22/6 (11)	5	106/52 (67)	7	
		18	7.9	4.0	7	29/14 (22)	16	130/63 (80)	10	34
		63	9.0	4.6	7	32/12 (22)		140/70 (93)	11	
12	Bronchiectasis	Basal	7.3			21/7 (14)		126/65 (86)	5	48
		Basal	7.0		1				5	48
		12	9.0		9				9	39
13	Bulbous emphysema	Basal	7.9	3.9		25/8 (15)		132/74 (90)	7	48
		7*	8.2	4.0		41/19 (28)		135/75 (95)	18	32
		53	9.0	4.4		39/15 (26)		140/76 (98)	15	31.5
		85	7.9	3.7					15	32
14	Post-tonsilitis	Basal	5.3	2.5		16/4 (10)		120/60 (80)	7	40.5
		7*	6.2	3.0		40/16 (26)		135/65 (85)	9	33
		60	5.6	2.7		31/11 (20)		135/72 (89)	9	35
15	Tension headache	Basal	7.0	3.9		19/4 (9)	2	138/76 (94)		44
		6	10.4	5.9		38/16 (27)				36
		8*	10.7	6.0		41/22 (31)		148/86 (110)		32
		33	10.9	6.1	9			165/98 (122)		33
16	Bronchiectasis	Basal	6.9	4.2	2			130/63 (88)		40
		9†	6.0	3.6	8			165/85 (110)		34
		39	6.4	4.2	7			155/82 (105)		33
		60	6.0	3.7	5			112/58 (74)		33

* After 1,500 ml. 6 per cent dextran.
† After 300 ml. 12 per cent salt-free dextran.

tients (Cases 8, 13, and 16, Table I). Two of these patients received 1,000 ml of 6 per cent dextran and the other patient received 300 ml. of 12 per cent dextran. Three other patients (Cases 6, 10, and 14) had increases of 15 per cent when cardiac output determinations were done within 20 minutes following end of infusion. In the remaining 10 patients, cardiac increases in output

ranged from 26 per cent to 53 per cent with an average increase of 44 per cent. A representative response (Case 9) is depicted in Figure 2. Previous results (1) indicated an increase of cardiac output in five of five patients; thus the total of the two reports indicates increases in cardiac output in 18 of 21 instances of plasma volume expansion. Data of the present investigation showed no correlation between the elevations of right auricular or pulmonary arterial pressure and the increase in cardiac output. The highest elevation in peripheral venous pressure was associated with no change in cardiac output (Case 13), whereas the smallest changes in peripheral venous pressures were accompanied by a 16 per cent and 30 per cent increase in cardiac output in two other patients (Cases 4 and 14). Of the three patients who received 1,500 ml. of 6 per cent dextran, only one (Case 15) showed a marked increase in cardiac output. This observation could be applied to all of the studies in that there was no correlation of the increase in cardiac output and the extent of plasma volume expansion.

Pulmonary arterial pressure increased in all eight patients studied (mean pressure increase, 11.5 ± 5.2). Both systolic and diastolic pulmonary arterial pressures increased in all instances, but the systolic pressure increased more than the diastolic. Right atrial pressure increased in all 10 patients studied, and the pressure remained elevated during the course of observation in 8 of 10 patients. The right atrial pressure was highest immediately following infusion and subsequently decreased toward pre-infusion levels. The right atrial pressure decreased more rapidly than did the pulmonary arterial pressure.

No significant association was found in the linear regression of the change from control of the cardiac output (liter per minute) (Y) on the mean right atrial pressure (mm. Hg) (X), (Slope $b = 0.010 \pm 0.146$, $p = > 0.1$). Similarly, when all cardiac output (Y) values are plotted against all right atrial pressures (X), no significant linear regression was observed (Slope $b = 0.55 \pm 0.092$, $p = 0.1$).

The pulmonary wedge pressure increased between 7 and 15 mm. Hg in five patients. Following dextran infusions, the gradient from pulmonary artery to pulmonary "capillary" showed no change in two patients and an increase of 2 to 4 mm. Hg in three patients. Pulmonary vascular resistance measured in five patients was found not to change significantly. Peripheral venous pressures were observed in eight patients. Immediately following infusion, this pressure increase (not listed in Table) exceeded 4.4 mm. Hg in all but one patient (Case 14) in which the pressure increased 2.5 mm. Hg. Three of four patients showed persistent moderate to marked elevations of peripheral venous pressure for over an hour. In the other patient, the venous pressure remained elevated for 25 minutes, then rapidly fell to below the initial pressure.

Femoral arterial mean pressure increased an average of 17 mm. Hg ± 3, $p = 0.001$ in all patients studied. The pulse pressure increased 8.3 mm. ± 3.3, $p = > 0.02$. Mean arterial pressures remained elevated one hour after infusion in all patients studied; in fact, arterial pressures at the end of one hour were often higher than immediate post-infusion levels. Femoral arterial pressure increases were, on the whole, no larger following 1,500 ml. than following 500 or 1,000 ml. Pulse rate was unchanged in eight patients following dextran infusion and six showed increases of 8 to 30 beats per minute. Changes in hematocrit following dextran infusions show that plasma volume expansion occurred in all patients and was maintained during the observation period. Other studies (7) have shown that the hematocrit gradually returns to normal over a period of five days following infusion of 6 per cent dextran.

DISCUSSION

In a previous study the administration of 500 ml. of 6 per cent dextran produced moderate elevations of pulmonary arterial pressures, while peripheral venous and systemic arterial pressure changes were small (1). In contrast, right atrial, pulmonary arterial and femoral arterial pressures were all elevated in the present study following the infusion of 1,000 and 1,500 ml. of dextran. The magnitude of the pulmonary pressure change in the presented data is greater than that in the peripheral veins and right auricle. Plasma protein infusions were previously reported to produce only transient increase in peripheral venous and arterial pressures (8), and the absence of pressure change was ascribed to generalized vasodilation which accommodated the expanded volume; however, these studies with dextran indi-

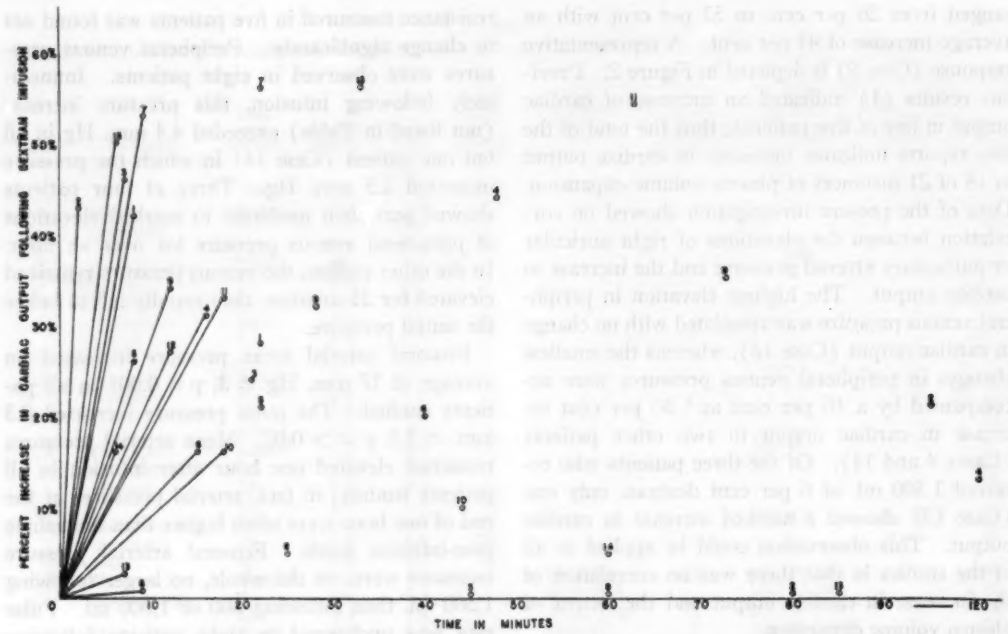

FIG. 1. PER CENT CHANGE IN CARDIAC OUTPUT FOLLOWING INFUSION OF 1,000 AND 1,500 ML. OF 6 PER CENT DEXTRAN

cate that the adjustment of vascular space to vascular volume is not maintained at control levels.

The data of Doyle, Wilson, Estes and Warren (9) and the findings of this study showed that comparable changes occur in pulmonary arterial and pulmonary wedge pressure. These data suggest that the left atrial, and possibly the left ventricular diastolic, pressure is elevated following plasma volume expansion. Although these changes might be suggestive of left ventricular failure, it would seem premature to speculate on this point before the performance of the left ventricle under increased work load is measured. In this regard, Morrison and Bloom (10) observed pulmonary hemorrhage rather than pulmonary edema as a result of maximal plasma volume expansion in dogs. Apparently, increased pulmonary vascular pressures as well as extreme hemodilution, anemia, and altered hemostasis produced this result.

The observation that cardiac output increased following hemodilution and hypervolemia confirmed the results of McMichael and Sharpey-Schafer (11). However, Warren, Brannon, Weens and Stead (12) indicated only an inconstant increase in cardiac output following plasma volume expansion with human albumin. It is possible that the conflict in the results of these experiments might be related to differences in materials used for plasma volume expansion, varying rates of administration of the expander, the degree of anemia from hemodilution (13), changes in peripheral resistance (14), as well as other unconsidered variables.

McMichael and Sharpey-Schafer (11) found increase in cardiac output and increase in right atrial pressure following rapid intravenous infusion of saline in six patients. In the present study, increase in right atrial pressure was frequently observed following plasma volume expansion with dextran; however, there was no significant association between increase in right atrial pressure and increase in cardiac output. These two experiments have demonstrated increase in right atrial pressure and increase in cardiac output following plasma volume expansion, but there was no evidence to show that the increase in cardiac output was related to atrial pressure changes. Despite

increased pressure in peripheral veins, right atrium, pulmonary artery, and pulmonary "capillary," as well as plasma volume expansion, the heart only shows a small increase in output in relation to its known capacity. Plasma volume expansion and the resulting hemodynamic changes have not been found to produce maximal changes in cardiac output.

CONCLUSIONS

Following intravenous administration of 1,000 to 1,500 ml. of 6 per cent dextran to normal individuals, there is prolonged plasma volume expansion. This increased plasma volume alters normal pressure-volume relationships in both the pulmonary and systemic vascular circuits, causing relatively greater increases in pressure in the pulmonary system. In some patients, accommodation to this plasma volume expansion might occur by generalized capillary and venous dilation, allowing the systemic venous and right atrial pressure to return toward normal levels. Cardiac output is usually increased, but this increase is not consistently associated with any measured pressure change in the venous or pulmonary system or the degree of plasma volume expansion. In contrast, a few patients with an expanded plasma volume may fail to increase cardiac output in spite of high right atrial and pulmonary arterial pressures.

SUMMARY

1. One thousand to 1,500 ml. of 6 per cent dextran in physiologic saline solutions were given in-

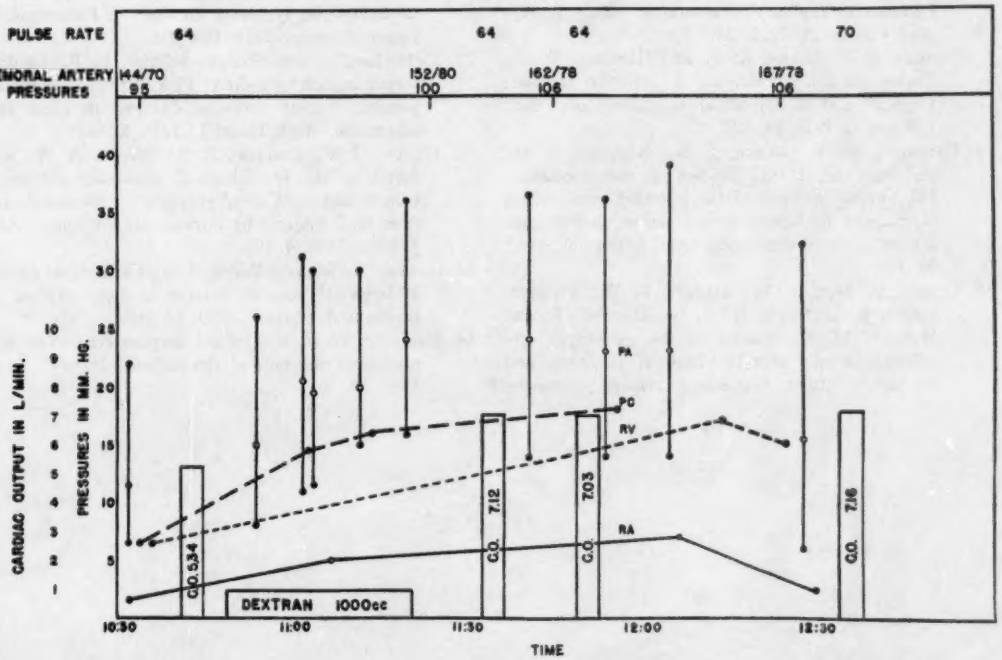

FIG. 2. HEMODYNAMIC CHANGE FOLLOWING THE INTRAVENOUS ADMINISTRATION OF 1,000 ML. OF 6 PER CENT DEXTRAN IN CASE 9

C. O. = Cardiac output
P. A. = Pulmonary arterial mean pressure
P. C. = Pulmonary "capillary" mean pressure
R. A. = Right atrial mean pressure
R. V. = Right ventricular pressure

Pulmonary arterial systolic and diastolic pressures are represented by the solid dots connected to the mean pulmonary arterial pressure representation.

travenously at the rate of 25 ml. per minute to 16 subjects, most of whom were convalescent.

2. Cardiac output increased 25 to 53 per cent in 10 patients, 15 per cent in three patients, and did not increase in three individuals.

3. Right atrial and pulmonary arterial pressure elevations increased markedly and were usually prolonged in all patients following dextran injections. The data indicate that increases in cardiac output cannot be related to increases in right atrial, pulmonary arterial or pulmonary wedge pressures.

REFERENCES

1. Witham, A. C., Fleming, J. W., and Bloom, W. L., The effect of the intravenous administration of dextran on cardiac output and other circulatory dynamics. J. Clin. Invest., 1951, **30**, 897.
2. Hellems, H. K., Haynes, F. W., and Dexter, L., Pulmonary 'capillary' pressure in man. J. Applied Physiol., 1949, **2**, 24.
3. Kinsman, J. M., Moore, J. W., and Hamilton, W. F., Studies on the circulation. I. Injection method: Physical and mathematical considerations. Am. J. Physiol., 1929, **89**, 322.
4. Hamilton, W. F., Moore, J. W., Kinsman, J. M., and Spurling, R. G., Studies on the circulation. IV. Further analysis of the injection method, and of changes in hemodynamics under physiological and pathological conditions. Am. J. Physiol., 1932, **99**, 534.
5. Dexter, L., Dow, J. W., Haynes, F. W., Whittenberger, J. L., Ferris, B. G., Goodale, W. T., and Hellems, H. K., Studies of the pulmonary circulation in man at rest. Normal variations and the interrelations between increased pulmonary blood flow, elevated pulmonary arterial pressure, and high pulmonary "capillary" pressures. J. Clin. Invest., 1950, **29**, 602.
6. Gibson, J. G., 2nd, and Evans, W. A., Jr., Clinical studies of the blood volume. I. Clinical application of a method employing the Azo dye "Evans Blue" and the spectrophotometer. J. Clin. Invest., 1937, **16**, 301.
7. Bloom, W. L., Present status of plasma volume expanders in the treatment of shock: clinical laboratory studies. Arch. Surg., 1951, **63**, 739.
8. Wilson, J. R., Jr., and Harrison, C. R., Cardiovascular, renal, and general effects of large, rapid plasma infusions in convalescent men. J. Clin. Invest., 1950, **29**, 251.
9. Doyle, J. T., Wilson, J. S., Estes, E. H., and Warren, J. V., The effect of intravenous infusions of physiologic saline solution on the pulmonary arterial and pulmonary capillary pressure in man. J. Clin. Invest., 1951, **30**, 345.
10. Morrison, J. L., and Bloom, W. L., The effect of continuous infusion of hydrolyzed dextran on the cardiovascular system of the dog. J. Pharmacol. & Exper. Therap., 1951, **103**, 355.
11. McMichael, J., and Sharpey-Schafer, E. P., Cardiac output in man by a direct Fick method. Effects of posture, venous pressure change, atropine, and adrenaline. Brit. Heart J., 1944, **6**, 33.
12. Warren, J. V., Brannon, E. S., Weens, H. W., and Stead, E. A., Jr., Effect of increasing the blood volume and right atrial pressure on the circulation of normal subjects by intravenous infusions. Am. J. Med., 1948, **4**, 193.
13. Gowdey, C. W., and Young, I. E., Cardiorenal effects of large infusions of dextran in dogs. Canad. J. Biochem. & Physiol., 1954, **32**, 559.
14. Hamilton, W. F., Role of the Starling concept in regulation of the normal circulation. Physiol. Rev., 1955, **35**, 161.

SKELETAL MUSCLE ANALYSES IN HEALTH AND IN CERTAIN METABOLIC DISORDERS. I. THE METHOD OF ANALYSIS AND THE VALUES IN NORMAL MUSCLE[1]

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Tissue analyses are of special importance in regard to the meager information available today concerning the role of magnesium in disease. Prior investigations of the concentration of serum magnesium have revealed modest fluctuations unassociated with any clear-cut, consistent, clinical syndromes (1–11). Balance studies (12–15) have been hampered by the lack of low magnesium diets with the result that fluctuations in the balance have been obscured by an excessive magnesium intake and excretion. This mineral is located almost exclusively in the cells or in the skeleton, and calculations based on a standard reference (16) for the composition of the body tissues and on a generally accepted partition (17) of the anatomical compartments of the body indicate that of the 2,100 milliequivalents of magnesium present in the adult body 45 per cent are located in the skeleton. Of the remaining 1,150 milliequivalents 98 per cent exist within the cells, and only 2 per cent of the extra-skeletal magnesium are located in the extracellular fluid.

This paper records the application of established methods of tissue analysis in a series of control muscles to determine the normal composition of skeletal muscle and its variations and also records the application of statistical techniques to define the precision of the methods. In a following report (18) a variety of muscle analyses in diseased states are compared with the control data to extend our information concerning the stability of intracellular magnesium. The authors are indebted to the late Professor J. L. Lilienthal and to Professor A. B. Hastings for the fundamental biochemical concepts and to Sir R. A. Fisher for the pertinent statistical techniques upon which these studies are based.

METHOD

Selection of skeletal muscle. This tissue was selected for analysis because it represents the greatest bulk of protoplasm, because of the accumulated information concerning its composition, and because of its availability at almost any operation.

Definition of the intra- and extracellular spaces: A biochemical dissection. In the absence of an infallible technique for distinguishing between the intracellular phase and extracellular phase we have accepted the chloride space as an approximation of the extracellular space. It is well recognized that in diseased states where chloride ions may penetrate into the muscle fiber cell, the extracellular space will be falsely enlarged. However, the chloride space still remains a most reasonable and practical means of correcting for the extracellular components to permit calculation by difference of the intracellular components. The evidence for the extracellular location of the chloride ion and for the proportionality of its total mass to the extracellular space has been recently reviewed (19).

Bases of reference in expressing the concentrations of the muscle components. In tissue analyses advantages exist in expressing the results in terms of more than one reference base. Changes in the concentrations of the components in relation to one base might not appear in relating the concentrations to another due to a concomitant shift of the second base. Other considerations in the selection of a reference base are the effects of analytical variations in its determination which would cause fluctuations devoid of any biological significance and the natural variation of a reference base which should be as restricted as possible to minimize the confusion this would introduce into the interpretations. These independent characteristics may be assessed by statistical techniques outlined below.

The reference base of intracellular water introduced by Hastings and Eichelberger (20) has been justified by water being the most plentiful constituent of tissues and by the success achieved in unfolding the properties of blood as a physiochemical system approximating a simple aqueous solution. More recently the use of non-collagen nitrogen (NCN), proposed by Lilienthal, Zierler, Folk, Buka, and Riley (21), as a reference base has the advantage of relating the intracellular components to a standard that is independent of any assumptions that the use of the chloride space entails in the calculation of the intracellular water. The non-collagen nitrogen, which is defined as the nitrogen in whole wet muscle fiber soluble in dilute alkali (0.05 N), is considered to be a measure of the functioning mass of muscle cells. The use of the solubilities of the muscle fibre proteins to separate

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them from the structural proteins of collagen and elastin has been reported by Lowry, Gilligan, and Katersky (22). These two reference bases of intracellular water and non-collagen nitrogen have been selected for the tissue analyses.

The details of the analysis adopted from the method of Lilienthal (21) are presented briefly to indicate any significant departures from the previously published techniques.

Physical preparation of the muscle and alkaline digestion. The samples of human muscle were excised during an operation under spinal or ether anesthesia from the edge of the incision or from the normal muscle of a freshly removed specimen. They were handled expeditiously and were protected against the loss of water by covering with a damp gauze. The samples weighed approximately 6 to 8 grams for a single analytical run, or twice this weight for duplicate runs. At the time of division and weighing of the muscle samples, gross connective tissue and fat were removed. Where duplicate samples were taken, homogeneous strips of muscle were bisected. The sampling error depends only on the gross evaluation of the muscle, and care was taken to diminish this source of variation.

The following portions were set aside:

One gram weighed into a previously tared, glass-stoppered Erlenmeyer flask for the determination of the water and fat content (20);

Six grams weighed into a previously tared weighing bottle for subsequent homogenization; and

One gram of muscle fixed in a formalin solution for histological study.

After the muscle had been weighed, the specimen was often frozen and stored pending further analysis. The blood for analysis was obtained just before or just after the excision of the muscle specimen, and a short series of paired determinations revealed no significant alterations in the serum values over this period of time. Infusions were virtually stopped well before obtaining a sample, and blood was not removed from an extremity into which an intravenous solution had been infused.

The largest portion of muscle was homogenized in a lucite Potter-Elvehjem homogenizer. A "pea soup" homogenate in distilled water was transferred to a 50-ml. volumetric flask, made up to volume, and designated as the *aqueous homogenate.*

Aliquots of the aqueous homogenate were treated with 2.5 volumes of dilute alkali to achieve a final concentration of 0.05 N NaOH or 0.05 N KOH. These sodium and potassium alkaline digests stood at room temperature for 18 hours prior to centrifugation.

The digests were centrifuged for one hour in the cold at a temperature of 4° to 8° C. Centrifugation under 1,400 to 1,600 G separated three distinct zones—a thin top layer of fat; an intermediate, aqueous solution containing the electrolytes and the non-collagen nitrogen compounds; and a bottom layer consisting of fine, particulate matter containing the insoluble, collagenous structural proteins. The middle layer was removed by pipette and designated as the *sodium* or *potassium non-collagen nitrogen (NCN) solution.* The separation of the fat from the NCN solution after approximately a twenty-fold dilution did not significantly alter the concentrations of the other components in the supernatant since the relative amount of fat present was reduced to about 1 per cent or less. Furthermore, the separation of the fat at this stage obviated the necessity of making a correction for its possible contribution to the NCN as proposed by others (23).

Chemical analyses. The methods were the same for blood and muscle specimens except for the dilutions employed, and the techniques were essentially those stated in the original publications with minor modifications to conform to the equipment and experience of the laboratory.

(a) Blood: Hematocrit (24), total nitrogen (25), serum sodium (26), serum potassium (26), serum inorganic phosphorus (27), chloride (28), serum magnesium (29), and serum water by drying to constant weight.

(b) Muscle: Aqueous homogenate—Magnesium in quadruplicate. Sodium NCN solution—Nitrogen and potassium in triplicate; phosphorus and chloride in quadruplicate. Potassium NCN solution—Sodium in triplicate.

Calculation of the derived values. The calculations for the derived values are symbolized below starting with the analytical values found and ending with the derived intracellular concentrations of a component expressed as the amount per 100 Gm. wet fat-free muscle, per kilogram of intracellular water, and per Gm. of NCN. The corrections for the chloride content and for the nitrogen content of the occluded blood in the muscle samples require preliminary explanation.

The chloride content of the red cells is an appreciable amount of the total chloride in whole muscle if the blood naturally saturating a fresh specimen is not removed by pressure and blotting. Preliminary studies using radioactive iodinated serum albumin compared the specific activity of the peripheral serum and the activity of the muscle sample. For the approximate calculation of the blood volume in the muscle sample it was assumed that the hematocrit in normal muscle was equal to the peripheral hematocrit of 45 per cent. The serum content of muscle was found to be 5 per cent by weight; and therefore, each 100 Gm. of muscle contains approximately 5 ml. of serum and 4.09 ml. of red cells. The correction for the chloride content of the red cells is obtained by multiplying the average red cell volume of 4.09 ml. per 100 Gm. of muscle by a factor to correct for any departure of the hematocrit from 45 per cent and by the concentration of chloride normally found in red cells—50 mEq. per L. (30). This red cell chloride correction may amount to 5 to 10 per cent of the total whole muscle chloride.

The nitrogen content of whole blood is an appreciable amount of the total NCN in whole muscle. This correction is obtained by multiplying the average whole blood volume of 9.09 ml. per 100 Gm. of muscle by the concentration of nitrogen found in the peripheral blood, and this may amount to 5 to 10 per cent of the total whole muscle NCN.

In the calculations, the Gibbs-Donnan factors tabu-

lated by Manery (19, pp. 349–350) were employed, and the factor of 0.977 appears below in the expression for the chloride space. In general, only sodium required an allowance for its extracellular location, and it has been used in the sample calculations below. For potassium, magnesium, and phosphorus the total amount present in wet fat-free muscle was usually employed where $(Na)_{IC}$ appears in the third part of the following calculations:

1. *Analytical values*

Whole wet muscle

$(H_2O)_m$ = Per cent of water by weight
$(Fat)_m$ = Per cent of fat by weight
$(Na)_m$, $(K)_m$, $(Mg)_m$, and $(Cl)_m$ = mEq. per 100 Gm.
$(P)_m$ = mM per 100 Gm.
$(NCN)_m$ = Gm. per 100 Gm.

Blood or serum

$(H_2O)_s$ = Gm. per 100 ml. serum
$(Na)_s$, $(K)_s$, $(Mg)_s$, and $(Cl)_s$ = mEq. per L. serum
$(N)_b$ = Gm. per 100 ml. whole blood
$(Hct)_b$ = Hematocrit in per cent

Factor converting whole wet muscle values to wet fat-free muscle values:

$$\frac{100}{100 - (Fat)_m}$$

Factor converting values per liter of serum to values per kilogram of serum water:

$$\frac{100}{(H_2O)_s}$$

2. *Derived values of chloride space, intracellular water, and corrected NCN*

Chloride in red cells (in mEq. per 100 Gm. of wet fat-free muscle):

$$(Cl)_{RC} = 4.09 \times \frac{(Hct)_b}{45} \times \frac{50}{1{,}000}$$

Chloride space (in Gm. H_2O per 100 Gm. wet fat-free muscle):

$$(Cl)\ Space = \frac{(Cl)_m \times \frac{100}{100 - (Fat)_m} - (Cl)_{RC}}{\frac{(Cl)_s}{0.977} \times \frac{100}{(H_2O)_s} \times \frac{1}{1{,}000}}$$

Intracellular water (in Gm. per 100 Gm. wet fat-free muscle):

$$(H_2O)_{IC} = (H_2O)_m \times \frac{100}{100 - (Fat)_m} - Cl\ space$$

Nitrogen in whole blood (in Gm. per 100 Gm. wet fat-free muscle):

$$9.09 \times \frac{(N)_b}{100}$$

Corrected NCN (in Gm. per 100 Gm. wet fat-free muscle):

$$(NCN)_s = (NCN)_m \times \frac{100}{100 - (Fat)_m} - 9.09 \times \frac{(N)_b}{100}$$

3. *Derived values of intracellular sodium, potassium, magnesium, and phosphorus*

(Sodium used as an example of the allowance necessary for the extracellular location. For the other intracellular components the total amount present in wet fat-free muscle was usually employed where $(Na)_{IC}$ appears below.)

Extracellular sodium (in mEq. per Gm. wet fat-free muscle):

$$(Na)_{EC} = \frac{(Na)_s}{1{,}000} \times \frac{100}{(H_2O)_s} \times \text{Gibbs-Donnan factor} \times \text{Cl space}$$

Intracellular sodium:

In mEq. per 100 Gm. wet fat-free muscle:

$$(Na)_{IC} = (Na)_m \times \frac{100}{100 - (Fat)_m} - (Na)_{EC}$$

In mEq. per Kg. intracellular water:

$$(Na)_{IC} \times \frac{1{,}000}{(H_2O)_{IC}}$$

In mEq. per Gm. intracellular NCN:

$$(Na)_{IC} \times \frac{1}{(NCN)_s}$$

RESULTS

Analytical data

In establishing the values of the components in normal muscle, the ten patients listed in Table I had two samples of grossly normal muscle removed from the excised surgical specimen. These twenty samples were analyzed in replicate for sodium, potassium, magnesium, phosphorus, chloride, and nitrogen. The fat and water content were determined by one analysis apiece in each sample. Except for the fat and water content, the muscle data in Table II are mean values of the replicate muscle sample analyses. These results are expressed directly per 100 grams of wet fat-free muscle except for the values of the fat which are expressed per 100 grams of whole muscle. These analyses establish the normal values of muscle in this study and provide the basic data from which the derived values are computed. Only one blood sample was obtained in conjunction with each patient, and the mineral, nitrogen, and hematocrit determinations were applied to both muscle samples. The precision of the method is reviewed in the following analysis of variance of the muscle data in Table II.

TABLE I

Patients providing control muscle samples

Patient	Unit number	Age	Sex	Diagnosis	Anesthesia	Muscle
1	902 921	61	F	Ca breast	GOE	Pectoralis, uninvolved in ca
2	862 781	42	F	Ca breast	GOE	Pectoralis, uninvolved in ca
3	680 668	80	M	Ca thyroid	GOE	Sternocleidomastoideus, uninvolved in ca
4	404 794	79	M	Ca mouth	GOE	Sternocleidomastoideus, uninvolved in ca
5	706 130	51	M	Arterial insufficiency, distal foot	Spinal	Semitendinosus and biceps femoris
6	338 429	81	F	Arterial insufficiency, distal foot	Spinal	Gastrocnemius and soleus
7	602 894	71	F	Arterial insufficiency, distal foot	Spinal	Semitendinosus and biceps femoris
8	6 194	50	F	Diabetic gangrene, distal foot; diabetes compensated	Spinal	Gastrocnemius and soleus
9	906 321	77	M	Diabetic gangrene, distal foot; diabetes compensated	Spinal	Semitendinosus and biceps femoris
10	866 726	70	F	Diabetic gangrene, distal foot; diabetes compensated	Spinal	Semitendinosus and biceps femoris

Analysis of variance of the analytical data

To account for the range of values of any muscle component noted by inspection in Table II, three separate sources of variation may be identified. The over-all variation is the net effect of variation introduced by 1) differences inherent in the composition of muscle between individual patients, 2) a lack of similarity in the two muscle samples obtained from a single patient, and 3) discrepancies in the analytical techniques. Since the first source of variation is the only one of clinical interest, it is necessary to assign to each

TABLE II

Analytical values of 10 control patients

Patient	Muscle sample	Per 100 Gm. wet fat-free muscle							Whole muscle	Per Kg. serum water		Whole blood	
		Na	K	Mg	P	Cl	NCN	H_2O	Fat	Na	Cl	N	Hct.
		mEq.	*mEq.*	*mEq.*	*mM*	*mEq.*	*Gm.*	*Gm.*	*Gm./ 100 Gm.*	*mEq.*	*mEq.*	*Gm./ 100 ml.*	%
1	I	2.63	8.51	1.40	5.18	1.71	2.49	80.2	5.28	149	112	3.11	32.7
	II	2.93	8.58	1.56	5.25	1.96	2.52	78.1	5.47				
2	I	3.12	9.87	1.70	6.05	1.51	2.97	79.0	2.10	150	111	2.88	30.9
	II	2.92	9.87	1.59	5.87	1.39	2.88	78.4	1.56				
3	I	3.82	9.25	1.57	4.91	2.29	2.31	81.0	1.30	151	110	2.80	30.0*
	II	3.24	9.40	1.33	5.15	1.82	2.53	80.0	1.10				
4	I	2.92	10.06	1.69	5.29	1.76	3.03	78.8	1.19	146	113	2.99	27.8
	II	3.50	9.74	1.71	5.34	2.15	2.92	79.0	1.46				
5	I	5.33	7.95	1.79	5.97	3.12	2.46	80.5	18.3	148	114	2.89	30.7
	II	5.08	8.23	1.66	6.37	3.12	2.45	80.5	18.5				
6	I	5.28	9.56	2.13	5.74	2.10	2.56	80.2	19.6	169	102	3.11	43.1
	II	5.36	9.86	2.11	6.10	2.23	2.73	83.5	22.3				
7	I	4.66	9.40	1.60	5.18	1.95	2.63	79.8	2.70	147	108	3.12	44.3
	II	5.18	9.05	1.53	5.12	2.38	2.54	79.8	3.40				
8	I	5.65	7.34	1.17	4.13	3.91	2.17	85.0	4.61	168	109	2.70	25.2
	II	4.90	8.06	1.25	4.79	3.16	2.41	82.0	5.18				
9	I	5.22	8.75	1.66	4.84	2.45	2.18	80.0	4.10	140	98.6	2.74	36.8
	II	4.97	8.91	1.86	5.04	2.32	2.41	80.0	5.70				
10	I	5.45	10.6	1.70	5.67	2.44	2.75	80.4	12.3	146	100	2.82	37.1
	II	5.07	9.58	1.49	5.42	2.34	2.59	79.6	7.7				

* Assumed value.

TABLE III

Analysis of variance of analytical values

1 Component*	2 Source of variation	3 Degrees of freedom	4 Sum of squares	5 Mean square	6 Analyses per subclass	7 Variance	8 Standard deviation	9 Mean of patients	10 Coefficient of variation
									%
Na	Patients	9	61.9824	6.8869†	5.90	1.116			
	Samples	10	2.9981	0.2998†	2.95	0.102			
	Analyses	39	0.0054	0.0001	1.00	0.000			
	Totals	58	64.9859			1.218	1.10	4.36	25
K	Patients	9	33.3274	3.7030†	6.00	0.542			
	Samples	10	4.5117	0.4512†	3.00	0.150			
	Analyses	40	0.0416	0.0010	1.00	0.001			
	Totals	59	37.8807			0.693	0.832	9.13	9.1
Mg	Patients	9	3.8160	0.4240†	7.99	0.0457			
	Samples	10	0.5849	0.0585†	4.00	0.0143			
	Analyses	60	0.0891	0.0015	1.00	0.0015			
	Totals	79	4.4900			0.0615	0.248	1.63	15
Cl	Patients	9	27.4471	3.0497†	7.80	0.360			
	Samples	10	2.4010	0.2401†	3.90	0.060			
	Analyses	58	0.2839	0.0049	1.00	0.005			
	Totals	77	30.1320			0.425	0.652	2.31	28
P	Patients	9	19.254	2.139†	7.33	0.264			
	Samples	10	2.061	0.206‡	3.68	0.029			
	Analyses	54	5.442	0.101	1.00	0.101			
	Totals	73	26.757			0.394	0.628	5.37	12
NCN	Patients	9	2.7742	0.3082†	5.29	0.0528			
	Samples	10	0.2904	0.0290†	2.76	0.0100			
	Analyses	33	0.0458	0.0014	1.00	0.0014			
	Totals	52	3.1104			0.0642	0.253	2.58	9.8
H_2O	Patients	9	44.92	4.99†	2.00	2.44			
	Samples	10	11.09	0.11	1.00	0.11			
	Analyses								
	Totals	19	56.01			2.55	1.60	80.3	2.0
Fat	Patients	9	905.9536	100.66†	2.00	49.5			
	Samples	10	16.1303	1.61	1.00	1.6			
	Analyses								
	Totals	19	922.0839			51.1	7.15	7.19	99

* Na, K, Mg, and Cl are expressed in mEq.; P in mM; and H_2O and NCN are expressed in Gm., all per 100 Gm wet fat-free muscle. Fat is stated in Gm. per 100 Gm. whole wet muscle.

† The mean square ratio, F, reveals a probability of less than 1 per cent that these variations in the analyses or samples can account for the greater variation in the samples or patients respectively by random sampling.

‡ The mean square ratio, F, reveals a probability of less than 5 per cent.

source its contribution to the total variation so that the differences noted in Table II are demonstrated to be not merely an accident of the complex analytical techniques or merely a result of random sampling of non-uniform skeletal muscle.

Table III presents the outline of the analysis of variance of the data following the principles of R. A. Fisher (31) and the details of the treatment by Snedecor (32). To conserve space the individual determinations have not been tabulated separately, and the sample means have already been presented in Table II. For example, the sodium data in Table III are based on 59 sodium analyses performed on twenty muscle samples

from ten patients. The two averages of the individual analyses for sodium in the two muscle samples obtained from Patient 1 are 2.63 and 2.93 according to Table II. The data in Table III on fat and water do not have their variation partitioned into three sources inasmuch as replicate analyses were not done. The first six columns contain the statistics on which is based the computation of the variance tabulated in Column 7. The variance totalled for each component in Column 7 is that of a single analysis in one sample from one patient. This variance is the appropriate one to determine the significance of subsequent analyses in diseased states that must perforce be based on less material than in these replicate studies. The standard deviations in Column 8 likewise are those to be assigned to a mean obtained from a series of patients providing one muscle sample each, on which only one analysis for a particular component is carried out. In short, the computed standard deviations have been corrected for the replications in the control data. In Column 9 are recorded the patient means which are calculated from the muscle data in Table II. The coefficient of variation in Column 10 will be considered in connection with the comparison of different reference bases.

As indicated in the footnote of Table III, the ratios of the mean squares tabulated in Column 5 reveal the highly probable circumstance that in all the determinations the variation of the analytical methods is not responsible for the variation in the samples and that the variation of the samples is not responsible for the variation in the patients. Indeed the variation introduced by the chemical methods employed is so slight in comparison to the main sources of variation from the muscle samples and patients that in the subsequent discussion of the derived data only the means of the sample analyses were employed in the calculations. We conclude that in every instance the analytical

TABLE IV

Derived values of 10 control patients

Patient	Sample	Per Kg. intracellular H_2O					Per Gm. NCN†				
		Na*	K	Mg	P	NCN†	Na*	K	Mg	P	H_2O‡
		mEq.	*mEq.*	*mEq.*	*mM*	*Gm.*	*mEq.*	*mEq.*	*mEq.*	*mM*	*Gm.*
1	I	10.5	128	21.2	78.0	33.3	0.317	3.85	0.636	2.34	30.1
	II	11.3	138	25.1	84.6	36.1	0.312	3.83	0.696	2.34	27.7
2	I	21.8	147	25.3	89.8	40.2	0.543	3.64	0.629	2.23	24.8
	II	20.8	146	23.6	86.8	38.8	0.538	3.76	0.606	2.24	25.8
3	I	18.7	148	25.2	78.6	33.0	0.568	4.48	0.762	2.38	30.3
	II	18.2	143	20.3	78.5	34.8	0.522	4.12	0.583	2.26	28.7
4	I	15.2	156	26.2	81.9	42.7	0.355	3.65	0.614	1.92	23.5
	II	17.9	158	27.8	86.8	43.2	0.416	3.68	0.646	2.02	23.2
5	I	32.4	145	32.6	109.0	40.0	0.810	3.61	0.814	2.71	25.0
	II	27.8	150	30.2	116.0	39.8	0.695	3.74	0.755	2.89	25.0
6	I	38.4	154	34.4	92.6	36.8	1.04	4.18	0.935	2.52	27.2
	II	35.4	154	33.0	95.4	38.3	0.926	4.02	0.863	2.49	26.1
7	I	38.6	147	25.0	81.0	36.8	1.05	4.00	0.681	2.21	27.2
	II	40.8	151	25.5	85.2	37.7	1.09	4.00	0.677	2.27	26.6
8	I	5.48	144	22.9	81.0	37.6	0.146	3.82	0.610	2.15	26.6
	II	10.5	145	22.5	86.4	38.9	0.268	3.73	0.578	2.22	25.7
9	I	42.7	144	27.2	79.4	31.7	1.35	4.53	0.680	2.50	31.5
	II	36.5	150	31.3	84.8	36.4	1.00	4.12	0.862	2.33	27.5
10	I	41.3	182	29.3	97.5	42.8	0.964	4.25	0.683	2.28	23.4
	II	37.0	164	25.5	92.7	39.9	0.927	4.11	0.640	2.33	25.0
Mean		26.1	150	26.7	88.3	37.9	0.692	3.96	0.707	2.33	26.5
Standard error		3.98	3.48	1.28	3.26	1.03	0.109	0.088	0.035	0.07	0.74
Fiducial limits of mean:											
P = 0.90		19–33	144–156	24–29	82–94	36–40	0.49–0.89	3.8–4.1	0.64–0.77	2.2–2.5	25–28
P = 0.99		13–39	139–161	23–31	78–99	35–41	0.34–1.05	3.7–4.3	0.59–0.82	2.1–2.6	24–29
Standard deviation		12.6	11.0	4.06	10.3	3.27	0.346	0.277	0.110	0.223	2.35
Coefficient of variation %		48	7.3	15	12	8.6	50	7.0	16	9.6	8.9

* The sodium values have been reduced by the amount in the chloride space.
† The NCN has been reduced by the amount of the nitrogen in the vascular space.
‡ Intracellular water.

TABLE V

Coefficients of variation of analytical and derived data *

	Component							
	Na	K	Mg	Cl	P	NCN	H_2O	Fat
Analytical data								
Per 100 Gm. wet, fat-free muscle (Tables II and III)	25	9.1	15	28	12	9.8	2.0	99
Derived data								
Per Kg. intracellular H_2O (Table IV)	48	7.3	15		12	8.6		
Per Gm. NCN (Table IV)	50	7.0	16		9.6		8.9	
Per 100 Gm. dry, fat-free solids (text)	49	10	17		11		12	

* All values are $\frac{\text{standard deviation} \times 100}{\text{mean}}$.

techniques were sufficiently precise to distinguish between samples and that in each patient the two muscle samples were representative and sufficiently similar to distinguish between the control patients. These conclusions and the calculation of the variance of a series of single analyses on single samples from different patients are indispensable for the future application of the control data.

In this analysis of variance, the possible source of variation based on different muscles in a patient having dissimilar composition has not been considered because the data collected are not extensive enough. If this source of variation exists, it is included here in that attributed to the individual patient.

Derived data

In Table IV the derived values of the intracellular components are expressed per kilogram of intracellular water and per gram of NCN. At the foot of the Table the mean, standard error, fiducial limits with $P = 0.90$ and $P = 0.99$, standard deviation, and the coefficient of variation are displayed. The fiducial limits were computed from the *t*-distribution (31), and 9 degrees of freedom were employed because of the comparisons between ten patients. The standard deviation was determined as in Table III, but is based on the duplicate samples from different patients inasmuch as the analytical variation had been shown in Table III to be inconsequential in comparison to that of the samples and patients.

If the data are expressed in terms of 100 Gm. dry fat-free solids, the following mean values are obtained: Na, 8.13 mEq.; K, 46.9 mEq.; Mg, 8.36 mEq.; P, 27.6 mM; NCN, 11.9 Gm.; H_2O, 314 Gm. The sodium and water have been corrected for the amount in the chloride space, and the NCN has been corrected for the nitrogen in the vascular space.

The chloride space per 100 Gm. wet fat-free muscle has a mean value of 19.3 Gm. with a standard deviation of 5.81 Gm. and a coefficient of variation of 30 per cent. These values and those in the preceding paragraph may be derived from Table II.

Comparison of reference bases

A useful statistic for comparing the uniformity in data is the coefficient of variation, and in Table V are set forth the coefficients assigned to the data expressed in relation to four different reference bases. A desirable characteristic of a reference base is that its use introduces a measure of uniformity in the data consistent with the reference base itself not undergoing wide fluctuations. In Table V the correction for the extracellular sodium to permit a calculation of the intracellular sodium increased the variation in the derived sodium data in comparison to the analytical data. The coefficient of variation of the sodium and chloride in the analytical data and that of the chloride space of 30 per cent are approximately equal because of their sharing a common anatomical compartment. The coefficient of variation for water in the analytical data has the low value of 2 per cent,

TABLE VI

Tissue analyses from the literature *

Author	Subject		Component per 100 Gm. wet, fat-free tissue						
			Na	K	Mg	P	Cl	H_2O	N
			mEq.	*mEq.*	*mEq.*	*mM*	*mEq.*	*Gm.*	*Gm.*
Cotlove *et al.* (33)	Rat	Mean	2.08	10.6	2.23	7.68	1.17	72.6	3.52
		S.E.	0.04	0.2	0.03	0.10	0.01	0.1	0.03
Lilienthal *et al.* (21)	Rat	Mean			2.35	6.97			3.33†
		S.D.			0.18	0.31			0.109
Hastings and Eichelberger (20)	Dog	Mean	3.24	8.21			2.15	76.5	
		S.D.	0.48	1.0			0.28	0.64	
Cullen *et al.* (34)	Man‡	Mean		6.29	1.51	4.84	3.13	77.4	
Shohl (16)	Man	Mean	3.1	9.3	1.9	7.1	1.8	79	
Mudge and Vislocky (35)	Man	Range in 3 controls	3.59–4.42	8.19–8.80			2.74–2.97		2.93–3.37
Baldwin *et al.* (23)	Man	Mean		9.46	1.64	5.90			2.70†
		S.D.		1.17	0.21	0.710			0.250
Barnes, Gordon, and Cope	Man	Mean	4.36	9.13	1.63	5.37	2.31	80.3	2.58†
		S.D.	1.10	0.832	0.248	0.628	0.652	1.60	0.253

* The standard error (S.E.) or standard deviation (S.D.) of the values are stated when given by the authors. They are not strictly comparable because of unstated differences in their computation.
† Value represents NCN.
‡ Autopsy material.

and it increases in the derived data because intracellular water is referred to rather than total water. Furthermore, the original expression of the analytical data in terms of the weight of wet tissue inevitably leads to greater uniformity in the water content.

In considering these coefficients of variation it might be supposed that if the results were expressed per kilogram of intracellular water or per gram of non-collagen nitrogen that a greater measure of uniformity might be achieved. Indeed the rationale for introducing these reference bases is founded on the assumption commonly stated, but never proved, that a greater uniformity would appear in the derived results. However, a comparison of the coefficients in Table V reveals that no striking increase in the uniformity is obtained by expressing the results related to the intracellular water, to the NCN, or to dry solids. Naturally, the fact that no greater uniformity is noted here does not exclude the possibility of more meaningful interpretations of pathological material by employing these same reference bases. This has been substantiated by further experience in diseased states (16).

Comparison of data with other analyses

The data reported here are in agreement with previous reports in the literature as displayed in Table VI. The close agreement between Baldwin, Robinson, Zierler, and Lilienthal (23), who first applied this method to clinical material, and the data of this paper confirms our impressions of the precision of the methods. The standard deviations as published in various reports are calculated by differing and unstated methods and refer to quantities that are not strictly comparable. However, they serve as a rough guide to the dispersion of the values and indicate that the data of this paper conform to previous tissue analyses.

SUMMARY

A composite scheme of tissue analysis based on the concepts introduced by A. B. Hastings and J. L. Lilienthal has been presented with details of the analytical methods as applied to skeletal muscle. The outline of the calculations for the derived values of the concentrations of sodium, potassium, magnesium, phosphorus, water, and non-collagen nitrogen is given. These concentra-

tions are referred to intracellular water derived by means of the chloride space and to the non-collagen nitrogen content of skeletal muscle.

Analytical and derived values from a control series of analyses from ten patients are reported together with a statistical evaluation of the results. Various reference bases are considered in regard to their property to diminish the dispersion of the data, and the similarity of the results with other tissue analyses is discussed.

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REFERENCES

1. Hirschfelder, A. D., and Haury, V. G., Clinical manifestations of high and low plasma magnesium. Dangers of epsom salt purgation in nephritis. J. A. M. A., 1934, **102**, 1138.
2. Hirschfelder, A. D., and Haury, V. G., Variations in plasma magnesium and potassium in epilepsy. Proc. Soc. Exper. Biol. & Med., 1935, **33**, 40.
3. Brookfield, R. W., The magnesium content of serum in renal insufficiency. Quart. J. Med., 1937, N.S. **6**, 87.
4. Hirschfelder, A. D., and Haury, V. G., Variations in magnesium and potassium associated with essential epilepsy. Arch. Neurol. & Psychiat., 1938, **40**, 66.
5. Haury, V. G., and Cantarow, A., Variations of serum magnesium in 52 normal and 440 pathologic patients. J. Lab. & Clin. Med., 1942, **27**, 616.
6. Haury, V. G., Variations in serum magnesium in health and disease: A review. J. Lab. & Clin. Med., 1942, **27**, 1361.
7. Dine, R. F., and Lavietes, P. H., Serum magnesium in thyroid disease. J. Clin. Invest., 1942, **21**, 781.
8. Martin, H. E., and Wertman, M., Serum potassium, magnesium, and calcium levels in diabetic acidosis. J. Clin. Invest., 1947, **26**, 217.
9. Edmondson, H. A., Berne, C. J., Homann, R. E., Jr., and Wertman, M., Calcium, potassium, magnesium and amylase disturbances in acute pancreatitis. Am. J. Med., 1952, **12**, 34.
10. Stutzman, F. L., and Amatuzio, D. S., Blood serum magnesium in portal cirrhosis and diabetes mellitus. J. Lab. & Clin. Med., 1953, **41**, 215.
11. Flink, E. B., Magnesium deficiency syndrome in man. J. A. M. A., 1956, **160**, 1406.
12. Bulger, H. A., and Gausmann, F., Magnesium metabolism in hyperparathyroidism. J. Clin. Invest., 1933, **12**, 1135.
13. Tibbetts, D. M., and Aub, J. C., Magnesium metabolism in health and disease. I. The magnesium and calcium excretion of normal individuals, also the effects of magnesium, chloride, and phosphate ions. J. Clin. Invest., 1937, **16**, 491.
14. Tibbetts, D. M., and Aub, J. C., Magnesium metabolism in health and disease. II. The effect of the parathyroid hormone. J. Clin. Invest., 1937, **16**, 503.
15. Tibbetts, D. M., and Aub, J. C., Magnesium metabolism in health and disease. III. In exophthalmic goiter, basophilic adenoma, Addison's disease and steatorrhea. J. Clin. Invest., 1937, **16**, 511.
16. Shohl, A. T., Mineral metabolism. Am. Chem. Soc. Monograph series 82. New York, Reinholt Publishing Co., 1939.
17. Edelman, I. S., Olney, J. M., James, A. H., Brooks, L., and Moore, F. D., Body composition. Studies in the human being by the dilution principle. Science, 1952, **115**, 447.
18. Barnes, B. A., and Cope, O., Skeletal muscle analyses in health and in certain metabolic disorders. II. Determinations in disease, In preparation.
19. Manery, J. F., Water and electrolyte metabolism. Physiol. Rev., 1954, **34**, 334.
20. Hastings, A. B., and Eichelberger, L., The exchange of salt and water between muscle and blood. I. The effect of an increase in total body water produced by the intravenous injection of isotonic salt solutions. J. Biol. Chem., 1937, **117**, 73.
21. Lilienthal, J. L., Jr., Zierler, K. L., Folk, B. P., Buka, R., and Riley, M. J., A reference base and system for analysis of muscle constituents. J. Biol. Chem., 1950, **182**, 501.
22. Lowry, O. H., Gilligan, D. R., and Katersky, E. M., The determination of collagen and elastin in tissues with results obtained in various normal tissues from different species. J. Biol. Chem., 1941, **139**, 795.
23. Baldwin, D., Robinson, P. K., Zierler, K. L., and Lilienthal, J. L., Jr., Interrelations of magnesium, potassium, phosphorus, and creatine in skeletal muscle of man. J. Clin. Invest., 1952, **31**, 850.
24. Peters, J. P., and Van Slyke, D. D., Quantitative Clinical Chemistry. Volume II, Methods. Baltimore, The Williams & Wilkins Company, 1932, p. 74.
25. Folin, O., Determination of non-protein nitrogen *in* Laboratory Manual of Biological Chemistry, 5th ed. New York, Appleton-Century, 1934, p. 265.
26. White, J. U., Precision of a simple flame photometer. Anal. Chem., 1952, **24**, 394.

27. Fiske, C. H., and Subbarow, Y., The colorimetric determination of phosphorus. J. Biol. Chem., 1925, **66**, 375.
28. Wilson, D. W., and Ball, E. G., A study of the estimation of chloride in blood and serum. J. Biol. Chem., 1928, **79**, 221.
29. Garner, R. J., Colorimetric determination of magnesium in plasma or serum by means of titan yellow. Biochem. J., 1946, **40**, 828.
30. Rapoport, S., Stevens, C. D., Engel, G. L., Ferris, E. B., and Logan, M., The effect of voluntary overbreathing on the electrolyte equilibrium of arterial blood in man. J. Biol. Chem., 1946, **163**, 411.
31. Fisher, R. A., Statistical Methods for Research Worker, 7th ed. Edinburgh, Oliver and Boyd, 1938.
32. Snedecor, G. W., Statistical Methods Applied to Experiments in Agriculture and Biology, 4th ed. Ames, Iowa, The Iowa State College Press, 1946, pp. 236–241.
33. Cotlove, E., Holliday, M. A., Schwartz, R., and Wallace, W. M., Effects of electrolyte depletion and acid-base disturbance on muscle cations. Am. J. Physiol., 1951, **167**, 665.
34. Cullen, G. E., Wilkins, W. E., and Harrison, T. R., Electrolytes in human tissue. II. The electrolyte content of hearts and other tissues from cases with various diseases. J. Biol. Chem., 1933, **102**, 415.
35. Mudge, G. H., and Vislocky, K., Electrolyte changes in human striated muscle in acidosis and alkalosis. J. Clin. Invest., 1949, **28**, 482.

CATION ACCUMULATION BY MUSCLE TISSUE: THE DISPLACEMENT OF POTASSIUM BY RUBIDIUM AND CESIUM IN THE LIVING ANIMAL [1, 2]

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The mechanism responsible for the selective accumulation of potassium by muscle cells is not known. A theory which has gained wide acceptance in recent years is that potassium passively diffuses into muscle cells along an electrical gradient maintained by the active extrusion of sodium (1–4). According to this view, the negative potential normally found inside the muscle cell is produced by this "sodium pump," and in turn causes the accumulation of potassium against a concentration gradient.

If sodium were the only cation transported actively by the muscle cell, and if the accumulation of potassium were entirely a matter of passive diffusion down an electrical gradient, then one would expect other univalent cations capable of penetrating cells to distribute themselves at equilibrium across the muscle cell membrane in the same concentration ratio as potassium. Rubidium and cesium, univalent alkali metals with physicochemical characteristics closely resembling those of potassium, would appear to be ideally suited for testing this hypothesis. These cations penetrate cells readily and can substitute for potassium in many biological processes (5).

In the experiments reported here an attempt was made to test the theory of passive uptake of potassium by comparing the equilibrium distribution of potassium with that of rubidium or cesium in the muscle tissue of growing rats fed equivalent, large quantities of these elements. Previous studies (6–9) have already established in a general way that animal tissues are capable of accumulating significant quantities of rubidium or cesium, but there have been no determinations of the tissue-plasma concentration gradients when organisms are fed non-tracer amounts of these substances and no quantitative measurements of the extent to which these ions are taken up by tissues relative to potassium. The results of the present work indicate a distinctly greater affinity of rat muscle tissue for rubidium and cesium than for potassium, and the data therefore suggest that processes more complex than simple diffusion are involved in the normal accumulation of potassium.

METHODS

Young male rats of the Sprague-Dawley strain weighing 100 to 150 grams were depleted of potassium and made alkalotic by the feeding of a potassium-free alkaline-ash diet (10) for a period of two weeks. At the end of this time the animals were divided into five groups. Group I, the *potassium-deficient controls* (12 animals), was sacrificed at the end of the two-week period of potassium-depletion. Group II, the *potassium-added controls* (11 animals), was maintained for two more weeks on the same diet but 20 mEq. per L. of KCl was added to the drinking water. Group III, the *two week rubidium* group (30 animals), was given 20 mEq. per L. of KCl plus 20 mEq. per L. of RbCl in the drinking water for a period of two weeks. Group IV, the *three-week rubidium* group (17 animals), was treated in the same way as Group III, but it was allowed to drink the KCl and RbCl mixture for three weeks. Group V, the *two-week cesium* group (17 animals), was given a mixture of 20 mEq. per L. of KCl and 20 mEq. per L. of CsCl in the drinking water for two weeks following the period of potassium depletion. In addition, there was a separate group of animals, Group VI, the *normal controls* (12 animals). These animals were *not* initially depleted of potassium, but were maintained for three weeks on the experimental diet, plus 20 mEq. per L. KCl in their

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TABLE I

Plasma concentration

Group	No.	Na	K	Rb	Cs	CO_2	Cl
		mEq./L.	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*
I. K-deficient controls	12	139.0 ±1.9	1.6 ±0.2			31.7 ±2.9	90.6 ±2.7
II. K-added controls	11	146.1 ±2.0	3.7 ±0.6			29.4 ±2.9	99.4 ±2.7
III. Two-week Rb	30	141.4 ±5.2	1.9* ±0.4	0.86 ±0.47		20.3 ±2.5	106.6 ±2.5
IV. Three-week Rb	17	140.4 ±3.5	1.2† ±0.4	0.83 ±0.20		18.4 ±3.2	108.5 ±3.8
V. Two-week Cs	17	144.9 ±4.8	2.0 ±0.7		0.55 ±0.11	24.1‡ ±3.5	101.4 ±4.3
VI. Normal controls	12	142.1 ±3.5	3.9 ±0.3			27.2 ±1.5	100.6 ±1.8

* Sixteen analyses.
† Four analyses.
‡ Seven analyses.

drinking water. The animals were allowed access to drinking water *ad libitum*, so that the exact intake of each of the added cations was not measured, but in every animal given rubidium or cesium plus potassium the intake of each of the two cations was always equal. The purity of all the rubidium and cesium salts used was always checked either by differential precipitation and weighing as the sulfate salt or by paper chromatography (11).

At the end of each experiment the animals were exsanguinated through the abdominal aorta, under light amytal anesthesia. The blood was immediately heparinized and the plasma separated by centrifugation under oil. Samples of skeletal muscle were dissected free of all obvious fat and connective tissue, minced, and then extracted in hot water. The completeness of extraction of all the muscle cations measured was verified by a comparison of this method with a dry-ashing technique. Plasma analyses for sodium, chloride and CO_2 content were carried out by flame photometry, modified Volhard titration, and standard manometric technique, respectively. Similar methods were applied to the determination of sodium and chloride in the aqueous extracts of muscle. In the control animals not given rubidium or cesium, determinations of potassium in the plasma and muscle extracts were carried out by flame photometry. In all other experiments in which plasma and muscle extracts contained mixtures of potassium and rubidium, or potassium and cesium, special techniques had to be used for the simultaneous analysis of these elements.

The procedure devised was a combination of chemical analysis and radioisotope-tracer technique. All rubidium or cesium used in any one experiment was labeled at the beginning of the experiment by the addition of radioactive rubidium 86[3] or cesium 134[3] to the drinking water. The purity of the radioactive tracers was checked in every instance by plotting decay curves. Since all cesium or rubidium had been added to the diet at a known and constant specific activity, it was possible to determine the concentration of rubidium or cesium in any sample simply by measurement of its radioactivity. The *total* content of potassium plus rubidium, or potassium plus cesium in the sample, was then determined by the method of chloroplatinate titration (12). This latter technique was found to give essentially complete recoveries of standard mixtures of potassium, rubidium and cesium in serum and in muscle extracts. Knowing the sum of the alkali ions and the content of rubidium or cesium, it was then possible to calculate potassium as the difference between these two values.

All the data reported here were obtained by the "combined" method described above. Later on it was found possible to use flame photometry for the analysis of certain potassium and cesium mixtures, and in a number of experiments analyses were simultaneously carried out by both methods and found to give satisfactory agreement. In a few experiments, not reported here, mixtures of the alkali metals were separated by paper chromatography (11), and then each ion was eluted separately in water and determined by flame photometry. Comparison of this method with the "combined" radioactive-chloroplatinate technique used in the present study also gave a satisfactory correlation (13).

Intracellular concentration was expressed in terms of "intracellular water," which was taken as the difference between total water and "extracellular water." Total muscle water was determined by drying whole wet tissue to constant weight. "Extracellular water" was calculated from the chloride space on the assumption that chloride was exclusively extracellular in position. A constant correction for fat of 1 per cent of whole wet muscle weight was applied to each tissue specimen.

[3] Obtained from Abbott Laboratories.

RESULTS

Most of the animals appeared to tolerate the feeding of rubidium or cesium very well for the first two weeks. Having virtually stopped their normal growth during the two-week preliminary period of potassium deficiency, the rats began to gain weight and grow again at an almost normal rate during the initial period of feeding the rubidium-potassium and cesium-potassium mixtures. However, after about two weeks signs of toxicity began to appear, consisting chiefly of neuromuscular irritability and cessation of growth. Attempts to continue the feeding of rubidium beyond three weeks and the feeding of cesium beyond two weeks resulted in a progressively higher mortality, with the animals usually dying in convulsions.

The analytical data are summarized in Tables I and II. In these Tables the figures given are the group means, plus or minus the standard deviation of the observed values. In each instance the number of analyses is equal, or nearly equal, to the number of animals in the group. The few instances in which the number of analyses is significantly less than the number of animals are appropriately indicated. These discrepancies were the result of random technical difficulties caused by the small quantities of material available for analysis. In every case, however, all analyses actually carried out are included in the mean.

In the ensuing discussion whenever differences between means are called "significant," it is meant that the "t-test" gives values of "P" less than 0.01. "Borderline significance" denotes "P" values greater than 0.01 but less than 0.05.

A. Plasma

The results of the plasma values are summarized in Table I. The data demonstrate the following points:

1. Two weeks on the K-deficient, alkaline-ash diet (Group I) resulted in the expected hypokalemic alkalosis with significant reductions in plasma concentration of potassium and chloride and a significant rise in plasma CO_2 content compared to the normal controls (Group VI). The mean values were: potassium, 1.6 mEq. per L.; chloride, 90.6 mEq. per L.; and CO_2, 31.7 mEq. per L.

2. Addition of 20 mEq. per L. KCl to the drinking water for two weeks (Group II) resulted in virtual disappearance of these changes. Although the mean plasma values for potassium (3.7 mEq. per L.), CO_2 (29.4 mEq. per L.), and chloride (99.4 mEq. per L.) were not quite back to normal, the differences from normal were not significant.

3. When potassium-depletion was followed by the addition of a mixture of KCl (20 mEq. per L.) and RbCl (20 mEq. per L.) to the drinking water

TABLE II

Muscle composition per liter intracellular water

Group	No.	Na	K	Rb	Cs	Σ_1*	Σ_2†	ICW‡
		mEq./L.	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*mEq./L.*	*ml./Kg. tissue*
I. K-deficient controls	12	60.7 ±11.6	107.1 ±20.9			107.1 ±20.9	167.8 ±18.9	600.0 ±14.3
II. K-added controls	11	10.0 ±8.8	168.8 ±12.1			168.8 ±12.1	178.4 ±6.4	598.8 ±12.7
III. Two-week Rb	30	4.2 ±2.6	75.4 ±11.7	90.7 ±11.8		166.1 ±8.0	170.2 ±7.3	609.3 ±11.6
IV. Three-week Rb	17	3.4 ±3.7	64.2 ±7.5	100.5 ±8.2		164.7 ±9.8	168.0 ±11.0	601.6 ±17.4
V. Two-week Cs	17	9.8§ ±5.6	75.1 ±8.1		96.8 ±9.7	171.9 ±12.2	182.4§ ±14.8	599.2 ±15.7
VI. Normal controls	12	6.4 ±3.9	171.1 ±8.2			171.1 ±8.2	177.4 ±5.1	625.8 ±22.8

* Σ_1 = K + Rb (or Cs).
† Σ_2 = K + Rb (or Cs) + Na.
‡ ICW = Intracellular water.
§ Eleven analyses only.

for two weeks (Group III) the low plasma potassium remained essentially unchanged (1.9 mEq. per L.), but there resulted a hyperchloremic acidosis, with a significant reduction in CO_2 and a significant rise in chloride, compared to both the normals (Group VI) and the K-added controls (Group II). The mean CO_2 was 20.3 mEq. per L., and the mean chloride 106.6 mEq. per L. Plasma rubidium was very low (0.86 mEq. per L.)—significantly lower than even the potassium concentration.

4. Continuation of the RbCl and KCl mixtures for a third week (Group IV) resulted in no definite further change in plasma composition, although there was a further reduction in CO_2 of borderline significance. The apparent further reduction in plasma potassium to 1.2 mEq. per L. may not be real since it was based on only four analyses.

5. Addition of a mixture of KCl (20 mEq. per L.) and CsCl (20 mEq. per L.) to the drinking water for two weeks following the initial period of potassium depletion (Group V) resulted in a reduction of CO_2 content significantly less than that produced by the KCl and RbCl mixtures. The CO_2 content (24.1 mEq. per L.), although significantly lower than in the K-deficient controls (Group I), was not significantly below the normal level (Group VI). Plasma potassium was low (2.0 mEq. per L.), and was not significantly different from the values in the rubidium groups (III and IV) or the K-deficient controls (Group I). Plasma cesium was only 0.55 mEq. per L., a value significantly lower than the simultaneous potassium concentration.

B. Muscle

Table II summarizes the results of the muscle analyses. The noteworthy findings were:

1. As expected intracellular potassium was low (107.1 mEq. per L.) and sodium high (60.7 mEq. per L.) in the K-deficient controls (Group I), as compared to the normal values in Group VI. There was a small but significant reduction in calculated intracellular water.

2. Addition of 20 mEq. per L. KCl to the drinking water for two weeks (Group II) virtually restored muscle composition to normal, there being no significant differences in intracellular Na and K between Groups II and VI. Intracellular water remained the same as in the K-deficient controls.

3. Addition of 20 mEq. per L. each of RbCl and KCl to the drinking water of the K-deficient rats resulted, after two weeks, in striking changes in muscle composition (Group III). Potassium was significantly reduced still further to a concentration of 75.4 mEq. per L. (less than half of normal) and sodium was lowered to 4.2 mEq. per L. Intracellular rubidium was 90.7 mEq. per L. (significantly higher than potassium) and the total measured intracellular cation (Σ_2) was normal. Intracellular water was essentially the same as in Groups I and II.

4. Continuation of the RbCl and KCl mixture for a third week (Group IV) resulted in a slight further drop in cellular potassium (64.2 mEq. per L.) and sodium (3.4 mEq. per L.), as well as a further rise in cellular rubidium (100.5 mEq. per L.). The difference in potassium content between the two- and three-week muscles was significant, but the difference in rubidium content was only of borderline significance.

5. Two weeks of treatment with a mixture of CsCl and KCl (Group V) lowered intracellular potassium to 75.1 mEq. per L. and sodium to 9.8 mEq. per L. Intracellular cesium was 96.8 mEq. per L. (significantly higher than the simultaneous potassium levels), but total cation and intracellular water were essentially the same as in the K-added and K-deficient controls.

C. Concentration ratios

Table III summarizes the mean distribution ratios for potassium, rubidium and cesium. As shown in the third column, the normal ratio of intracellular to extracellular potassium was 44.2 ± 4.1 (Group VI). Potassium deficiency caused a slight but significant elevation in this ratio to 67.7 ± 16.2. In the two-week rubidium group (III) and in the cesium-treated group (V) the ratio of intracellular to extracellular potassium concentration was restored to normal. (No ratio is given for Group IV because simultaneous plasma and muscle potassium values were available for only one animal.) The $\frac{\text{intracellular}}{\text{extracellular}}$ ratios for rubidium were 118.4 ± 30.2 and 116.2 ± 19.7 in the two- and three-week groups, respectively. The ratio for cesium was even higher than this, with a mean value of 185.2 ± 36.0. In the

TABLE III

Concentration ratios

	$Rb\ (or\ Cs)_M/K_M$*	$Rb\ (or\ Cs)_M/Rb\ (or\ Cs)_P$†	K_M/K_P‡	"R"§
I. K-deficient controls			67.7 ±16.2	
II. K-added controls			45.7 ±4.9	
III. Two-week Rb	1.23 ±0.34	118.4 ±30.2	41.0 ±7.9	3.11 ±1.05
IV. Three-week Rb	1.61 ±0.25	116.2 ±19.7	‖	2.75¶ ±0.37
V. Two-week Cs	1.33 ±0.21	185.2 ±36.0	42.5 ±9.3	4.61 ±0.81
VI. Normal controls			44.2 ±4.1	

* Ratio of rubidium (or cesium) concentration in muscle to that of potassium.
† Ratio of rubidium (or cesium) concentration in muscle to that in plasma.
‡ Ratio of muscle potassium concentration to plasma potassium concentration.
§ Ratio of the two ratios in columns two and three, *i.e.*, $\frac{Rb\ (or\ Cs)_M}{Rb\ (or\ Cs)_P} \div \frac{K_M}{K_P}$.
‖ Omitted because only one simultaneous analysis of K_M and K_P available.
¶ Four sets of analyses only.

last column of Table III is given the proportion of the rubidium (or cesium) ratio to the simultaneous potassium ratio (*e.g.*, $\frac{Rb_M}{Rb_P} \div \frac{K_M}{K_P}$, where the subscripts M and P denote concentration in muscle water and plasma, respectively). This value, called "R" in the table, gives an estimate of the relative degrees to which the various alkali ions are concentrated by the muscle. Thus it can be seen that rubidium is concentrated approximately 3 times as much as potassium, while cesium is concentrated 4.6 times as much as potassium.

DISCUSSION

The foregoing observations confirm and extend the earlier data which had indicated that rubidium and cesium could be taken up in large quantities by muscle cells (6–9). They also confirm the previously reported fact that rubidium chloride lowers the blood CO_2 content in alkalotic potassium-deficient rats (9) and extend the observations to demonstrate a similar, but less marked, acidifying effect of cesium chloride. Most significant of all, however, is the evidence presented here that despite very low plasma concentrations of these ions, muscle tissue may accumulate rubidium or cesium up to concentrations 20 to 60 per cent higher than that of potassium. Intracellular potassium concentration may be depressed to extraordinarily low levels and, as shown by the absence of significant change in cellular water content (Table III), this reduction is due to displacement of potassium by the "foreign" alkali ions, rather than simply to intracellular shifts of water accompanying the penetrating ions. Additional evidence against any significant alterations in water distribution was provided by the fact that the water content of whole muscle did not vary significantly in any of the groups.

The concentrations of intracellular potassium given in Table II are considerably lower than the values usually found in potassium depletion alone (10). Actually, the mean values listed in the Tables do not indicate the maximum limits to the replacement of potassium by rubidium or cesium. In individual animals, intracellular rubidium concentrations as high as 120 mEq. per L. and potassium concentrations as low as 50 mEq. per L. were found. The highest intracellular cesium concentration observed in the cesium experiments was 110 mEq. per L., and the lowest potassium concentration 60 mEq. per L. Whether even these concentrations represent the limits of the replacement process is not known. Current experiments are designed to study this problem more closely.

Prior depletion of potassium apparently is not essential for this extensive accumulation of rubidium. In a small group of normal animals fed a mixture of rubidium and potassium in their drinking water for a period of four weeks changes in muscle composition virtually the same as those reported in Table II were found. A few analyses of muscle taken after two or three weeks suggested that in this experiment rubidium had not yet reached its maximum intracellular accumulation at these times. In the experiments on the animals initially depleted of potassium, on the other hand, tissue accumulation of rubidium appeared to be more rapid.

The similarity in composition between the rubidium-treated muscles in the two-week and three-week rubidium groups suggests that a near-equilibrium state had been attained by the end of the second week. Unfortunately, it was not possible to establish this point with certainty by treating animals for longer periods of time because after three weeks on rubidium, or only two weeks on cesium, the rats began to die in increasing numbers. However, even if the present data do not represent a true steady state, the relative chronicity of the experiments, as well as the relative homogeneity of the data, would strongly suggest that equilibrium must have been at least closely approximated. Significant deviations from the steady state caused by possible transient fluctuations in plasma rubidium or cesium concentration following ingestion of these ions would appear unlikely in view of the relatively small variations of the individual plasma values about the group mean. Individual animals were sacrificed at varying time intervals after their last feeding, and if large fluctuations in plasma level had occurred this would have been obvious from the scatter of the data.

The findings of greatest relevance to this study are the concentration ratios listed in Table III. In the rubidium- and cesium-treated groups, the intracellular/extracellular ratios for potassium remained normal at approximately 40 to 1, but the ratio for rubidium was approximately 3 times this value and the ratio for cesium was about 4.5 times the potassium ratio. It would thus appear that rat skeletal muscle accumulates the alkali metals at equilibrium in a definite order of preference, cesium > rubidium > potassium. Data obtained in a number of recent studies with radioactive tracer doses of rubidium (14–16) or cesium (17) appear to imply somewhat similar conclusions. In these tracer experiments, although no significant amount of intracellular potassium was displaced by the administered ions, the latter accumulated in muscle, red cells and most viscera at tissue plasma concentration ratios equal to, or greater than, those for potassium.

As already pointed out, according to the theory which suggests the passive accumulation of potassium along an electrical gradient established by the "sodium pump," one should expect similar concentration gradients at equilibrium for all three alkali ions, since they would be diffusing passively along the same electrical field. Differences in the mobility of the ions might effect the kinetics of the uptake process, but they would not change the steady-state concentration ratios. Thus, the large differences in gradient actually observed constitute evidence against the theory of passive accumulation. Although it is conceivable that different mechanisms transport each of the ions, the existence of a single process is strongly suggested not only by their close chemical similarity, but also by the fact that each ion appears to inhibit competitively the penetration of the other two into frog muscle (18), as well as into red blood cells (19, 20). The simplest conclusion consistent with the available data would seem to be that a single process is responsible for the cellular accumulation of potassium, rubidium and cesium and that this process is not simply a matter of passive diffusion.

If the passive diffusion theory is not valid for muscle, then at least two alternative explanations for the present results seem possible.

The first possibility is that there is an active transport system for which potassium, rubidium and cesium compete. This transport system might be directed inwards, with the order of increasing selectivity being potassium, rubidium, cesium, or the system might be directed outwards and have the reverse order of selectivity. It is also conceivable that the active transport system, whatever its directional orientation, is *not* selective. If this were the case, the differences in the relative accumulation of these ions could be the result of differences in rates of diffusion in the direction opposite to that of the active transport system.

The concept of active *inward* transport in muscle gains indirect support from recent studies with yeast (3, 21), green algae (22), and de-sheathed toad nerve fibers (23), which indicate the existence of an active inward transport system for potassium which can operate without the simultaneous efflux of sodium. No direct experimental evidence for the active and independent uptake of potassium by muscle has yet appeared, but this concept is at least consistent with the fact that movements of sodium and potassium in and out of muscle do not always occur simultaneously and in a fixed stoichiometrical relationship (3, 24, 25) as might be expected if potassium accumulation were entirely and directly dependent upon a "sodium pump." It has recently been pointed out that the concentration ratio for potassium in frog muscle somewhat exceeds that predictable simply from the transmembrane potential difference, further suggesting the possibility of some active chemical force responsible for potassium accumulation (26).

A second alternative to the "passive diffusion" theory is suggested by the analogy between the relative concentration gradients for the alkali metals observed in these experiments and the behavior of certain ion exchange resins, which selectively bind these ions in the same order of preference. Selective binding of cations by fixed negative charges inside the cell has already been proposed as a mechanism for the normal accumulation of intracellular potassium (27). This hypothesis gains some support from the recent observation that relatively high concentrations of potassium are apparently maintained in frog muscle which has been soaked in potassium-free Ringer solution and poisoned with cyanide plus iodoacetate (25). On the other hand, selective binding of cations within the cell would be expected to result in significant reductions in tissue osmotic activity as intracellular potassium is displaced by cations like rubidium or cesium, for which the fixed negative charges have a higher affinity. The absence of any evidence of water shifts in these experiments therefore indicates either that large-scale binding does not occur, or that there exist cellular mechanisms which maintain total osmotic constancy despite fluctuations in cation activity.

At present there is no experimental basis for a choice among these or any other alternatives to the passive diffusion theory of potassium uptake. Obviously, much more information is required before an adequate description of potassium transport in muscle cells can be given.

SUMMARY AND CONCLUSIONS

Chronic feeding of equivalent amounts of rubidium and potassium or cesium and potassium to young rats resulted in replacement of half or more of the muscle potassium by rubidium or cesium. The final tissue-plasma concentration ratios for rubidium were approximately 3 times that for potassium, and the ratio for cesium was more than 4.5 times the potassium ratio.

The results suggest that muscle cells accumulate the alkali metals in a definite order of preference: cesium > rubidium > potassium. This fact is construed as evidence against the normal accumulation of potassium solely by passive diffusion along an electrical gradient produced by a "sodium pump," and two alternative explanations are discussed which would appear to be more consistent with the present observations.

REFERENCES

1. Dean, R. B., Theories of electrolyte equilibrium in muscle. Biol. Symposia, 1941, 3, 331.
2. Krogh, A., Croonian Lecture: The active and passive exchanges of inorganic ions through the surfaces of living cells and through living membranes generally. Proc. Roy. Soc., London, s. B., 1946, 133, 140.
3. Conway, E. J., Some aspects of ion transport through membranes. Symposia Soc. Exper. Biol., 1954, 8, 297.
4. Steinbach, H. B., The regulation of sodium and potassium in muscle fibers. Symposia Soc. Exper. Biol., 1954, 8, 438.
5. Relman, A. S., The physiological behavior of rubidium and cesium in relation to that of potassium. Yale J. Biol. & Med., 1956, 29, 248.
6. Mendel, L. B., and Closson, O. E., The paths of excretion for inorganic compounds. III. The excretion of rubidium. Am. J. Physiol., 1906, 16, 152.
7. Mitchell, P. H., Wilson, J. W., and Stanton, R. E., The selective absorption of potassium by animal cells. II. The cause of potassium selection as indicated by the absorption of rubidium and cesium. J. Gen. Physiol., 1921, 4, 141.
8. Heppel, L. A., and Schmidt, C. L. A., Studies on the potassium metabolism of the rat during pregnancy,

lactation and growth. Univ. California Publ., Physiol., 1938, **8**, 189.

9. Relman, A. S., Roy, A. M., and Schwartz, W. B., The acidifying effect of rubidium in normal and potassium-deficient alkalotic rats. J. Clin. Invest., 1955, **34**, 538.
10. Cotlove, E., Holliday, M. A., Schwartz, R., and Wallace, W. M., Effects of electrolyte depletion and acid-base disturbance on muscle cations. Am. J. Physiol., 1951, **167**, 665.
11. Steel, A. E., Separation and identification of alkali metals on paper chromatograms. Nature, London, 1954, **173**, 315.
12. Kolthoff, I. M., and Sandell, E. B., Textbook of Quantitative Inorganic Analysis, 3rd ed. New York, The Macmillan Co., 1952, pp. 395–398.
13. Levinsky, N., Allen, J. M., Connors, H. P., and Relman, A. S., Unpublished data.
14. Love, W. D., Romney, R. B., and Burch, G. E., A comparison of the distribution of potassium and exchangeable rubidium in the organs of the dog, using rubidium^{86}. Circ. Research, 1954, **2**, 112.
15. Tyor, M. P., and Eldridge, J. S., A comparison of the metabolism of rubidium^{86} and potassium^{42} following simultaneous injection into man. Am. J. M. Sc., 1956, **232**, 186.
16. Kilpatrick, R., Renschler, H. E., Munro, D. S., and Wilson, G. M., A comparison of the distribution of ^{42}K and ^{86}Rb in rabbit and man. J. Physiol., 1956, **133**, 194.
17. Hood, S. L., and Comar, C. L., Metabolism of cesium-137 in rats and farm animals. Arch. Biochem. & Biophys., 1953, **45**, 423.
18. Ling, G. N., Selective cellular permeability according to the fixed charge hypothesis. Proc. Internat. Physiol. Congress, 1953, p. 566.
19. Solomon, A. K., The permeability of the human erythrocyte to sodium and potassium. J. Gen. Physiol., 1952, **36**, 57.
20. Burch, G. E., Threefoot, S. A., and Ray, C. T., The rate of disappearance of Rb^{86} from the plasma, the biologic decay rates of Rb^{86}, and the applicability of Rb^{86} as a tracer of potassium in man with and without chronic congestive heart failure. J. Lab. & Clin. Med., 1955, **45**, 371.
21. Rothstein, A., and Enns, L. H., The relationship of potassium to carbohydrate metabolism in baker's yeast. J. Cell. & Comp. Physiol., 1946, **28**, 231.
22. Scott, G. T., and Hayward, H. R.,, Evidence for the presence of separate mechanisms regulating potassium and sodium distribution in *ulva lactuca*. J. Gen. Physiol., 1954, **37**, 601.
23. Shanes, A. M., and Berman, M. D., Penetration of the desheathed toad sciatic nerve by ions and molecules. I. Steady state and equilibrium distributions. J. Cell. & Comp. Physiol., 1955, **45**, 177.
24. Cooke, R. E., Segar, W. E., Cheek, D. B., Coville, F. E., and Darrow, D. C., The extrarenal correction of alkalosis associated with potassium deficiency. J. Clin. Invest., 1952, **31**, 798.
25. Shaw, F. H., and Simon, S. E., The nature of the sodium and potassium balance in nerve and muscle cells. Australian J. Exper. Biol. & M. Sc., 1955, **33**, 153.
26. Harris, E. J., Transport and Accumulation in Biological Systems. New York, Academic Press, Inc., 1956, pp. 166–169.
27. Ling, G. N., The role of phosphate in the maintenance of the resting potential and selective ionic accumulation in frog muscle cells *in* Phosphorus Metabolism, Vol. 2, W. D. McElroy and Bentley Glass, Eds. Baltimore, The Johns Hopkins Press, 1952, p. 748.

PORTO-CAVAL AND PORTO-PULMONARY ANASTOMOSES IN LAENNEC'S CIRRHOSIS AND IN HEART FAILURE [1]

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Unsaturation of arterial blood for oxygen in patients with cirrhosis of the liver was first reported by Snell (1) and has been confirmed in this laboratory (2). This was found to be associated with a low partial pressure of oxygen in arterial blood in the absence of any defect of alveolar-capillary diffusion (3). These findings indicated an abnormally large venous admixture, attributable either to uneven ventilation-perfusion relationships or to anatomical shunts by-passing the lungs.

Anastomoses between the portal venous system and the superior caval system are known to be prominent in patients with hepatic disease and portal hypertension. Inasmuch as anastomoses between the pulmonary veins, bronchial veins, and azygos veins have also been demonstrated (4–8), it appeared possible that anastomotic channels between the portal venous system and the pulmonary venous system might exist. These, in the presence of portal hypertension, might lead to admixture of unsaturated portal venous blood to oxygenated pulmonary venous blood. This hypothesis was tested anatomically by means of injection of the portal venous system in human cadavers.

MATERIAL AND METHODS

In 20 human cadavers, prior to routine post-mortem examination, the portal vein was injected *in situ* with a radiopaque lead-gelatin mass, a modified Schlesinger mixture (9), which does not enter vessels less than 40 μ in diameter. Methyl-green dye was added to the mass, which was injected under pressure of 7 to 10 cm. Hg. In order to obtain optimal filling of the vascular tree under study, the following vessels were ligated (Figure 1): the portal vein at the porta hepatis, the superior and inferior mesenteric veins, the splenic vessels, and both venae cavae at the right atrium. The aorta and the pulmonary artery were ligated at their point of origin.

After the mass had hardened, the viscera were removed *en bloc*. In several instances radiograms of the block were made before dissection. Sketches and color photographs at successive stages of the dissection permitted subsequent analysis and comparison of the injected vessels.

In general, 400 ml. was the optimal amount of mass. In five cases (Nos. 1–3, 11, 17) less than 350 ml. of mass was injected. In Cases 2 and 3 the amount was adequate to fill the portal venous system and the injection was considered satisfactory, while in three cases (Nos. 1, 11, 17) it failed to fill the portal venous system but could not be extended for technical reasons. In Case 8, where death was due to rupture of esophageal varices, the mass entered the esophagus freely, preventing filling of any mediastinal collateral channels that may have existed.

The material was divided into three groups (Table I).

Group I consisted of 10 cases of cirrhosis of the liver. All cases in this group had clinical evidence of hepatic decompensation and ascites, which exceeded 5 liters in six cases. Pathologically the patients all had classical Laennec's cirrhosis except for Case 6 whose liver showed "healed acute yellow atrophy." This was the only patient without history of alcoholism.

Group II comprised six cases of heart failure. The etiology of heart disease is indicated in Table I. Significant chronic passive congestion of the liver was present in five cases, of which two showed central hemorrhagic necrosis of the liver (Nos. 12 and 16) and one (No. 15) had cardiac cirrhosis: a patient with mitral stenosis and tricuspid insufficiency who had been in right heart failure for several years.

Group III included four cases without evidence of functionally significant hepatic or cardiac disease. The liver was structurally normal in all cases. Focal myocardial fibrosis was present in Cases 17 and 19, and the coronary arteries of Case 17 showed some sclerosis.

RESULTS

Group I

In all cases of cirrhosis, peri-esophageal veins (Figures 2 and 3) were observed to be injected via the short gastric and coronary veins and extended

[1] Aided by grants from the Massachusetts Heart Association and the Life Insurance Medical Research Fund.

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TABLE I

Localization of indicator material after injection into the portal vein

Case no.	Age	Sex	Diagnoses*	Amt. mass. inj. *ml.*	Venous beds injected†						
					Esophageal						
					Submucosal *cm.* above cardia		Peri-esophageal	Azygos hemi-azyg.	Mediastinal	Bronchial	Pleuro-pericardial	Pulm. veins left atrium
					Group I: Cirrhosis							
1	72	M	Laennec's cirrh.	100	0		+	0	0	0	0	0
2	68	F	Laennec's cirrh.	200	++	1	++	0	0	0	0	0
3	42	M	Laennec's cirrh.	200	0		++	0	0	0	0	0
4	58	F	Laennec's cirrh.	400	++	15	++	++	++	+	++	0
5	64	M	Laennec's cirrh.	400	++		++	++	++	+	++	0
6	59	M	Healed yel. atrophy	400	++	4	++	++	++	0	++	++
7	24	M	Laennec's cirrh.	400	++	14	++	++	++	+	++	++
8	50	M	Laennec's cirrh.‡	400	+		++	0	0	0	0	0
9	71	M	Laennec's cirrh.	400	++	15	++	++	++	+	++	0
10	55	M	Laennec's cirrh.	400	0		++	++	++	0	++	0
					Group II: Congestive heart failure							
11	83	M	Acute myocarditis. ASHD.	150	0		0	0	0	0	0	0
12	72	F	Myocard. infarcts old and new. CHN. of liver	400	+	5	+	0	+	0	0	0
13	70	F	Massive pulm. emboli and infarcts. ASHD. CPC. of liver	400	0		+	+	+	+	+	0
14	84	M	Carcinoma of prost. ASHD. CPC. of liver	400	0		+	+	+	0	0	0
15	42	F	RHD. MS. TI. Cardiac sclerosis of liver	400	+	15	++	++	+	0	+	0
16	77	F	ASHD. CHN. of liver	400	0		++	++	++	+	+	0
					Group III: Normal liver. No heart disease							
17	80	M	Bronchopneumonia	150	+	5	0	0	0	0	0	0
18	86	F	Cerebral thrombosis	350	+	18	+	0	0	0	0	0
19	63	M	Polyarteritis nodosa	400	+	5	+	+	+	0	+	0
20	54	M	Carcinoma of lung	400	+	15	+	+	0	0	+	0

* ASHD. = Arteriosclerotic heart disease. CHN. = Central hemorrhagic necrosis. CPC. = Chronic passive congestion. MS. = Mitral stenosis. RHD. = Rheumatic heart disease. TI. = Tricuspid insufficiency.

† 0 = No injection; + = injected; ++ = prominently injected.

‡ Cause of death: Rupture of esophageal varices. Injection mass in lumen of esophagus and stomach.

above the level of the carina. These veins were dilated and tortuous. They anastomosed freely with mediastinal veins and, through these, with pleuro-pericardial veins and quite prominently with the azygos and hemi-azygos veins (Figure 3) and through these with the superior vena cava. This venous network was demonstrated whenever the amount of injection mass was adequate except in Case 8 where the mass entered the esophagus from ruptured submucosal varices.

In four of the six full injections the posterior mediastinal veins anastomosed freely with peribronchial venules at the tracheal bifurcation and, through these, with bronchial veins of the main-stem bronchi, most prominently about the right main-stem bronchus. On one occasion (No. 7) several large mediastinal veins communicated with the hilus of the right lung, and one was followed to its termination in a pulmonary vein (Figure 3).

In two of the fully injected specimens (Nos. 6 and 7) the mass was found in the pulmonary veins, left atrium and left ventricle, while the right ventricle and aorta were free of mass.

Submucosal esophageal veins were found to be injected in 7 of the 10 cirrhotics. They were dilated and tortuous, *i.e.*, varicose, in six of these

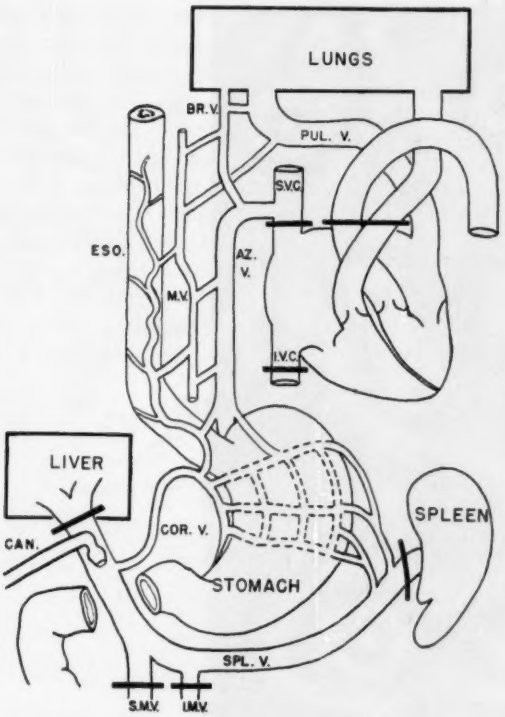

FIG. 1. SCHEMATIC REPRESENTATION OF THE PLAN OF INJECTION, SHOWING THE PRINCIPAL COLLATERAL CHANNELS IN THE PORTAL, MEDIASTINAL AND PULMONARY VENOUS BEDS

The heavy straight lines indicate ligatures.

Can., injection cannula; Eso., esophagus with peri-esophageal veins; M.V., mediastinal veins; Br.V., bronchial veins; Cor.V., coronary vein; Pul.V., pulmonary vein; S.V.C., superior vena cava; I.V.C., inferior vena cava; S.M.V., superior mesenteric vein; I.M.V., inferior mesenteric vein; Spl.V., splenic vein; Az.V., azygos vein.

(Figure 2), but were poorly filled in the seventh, who showed a ruptured esophageal varix (No. 8). These veins showed great variation, extending from 1 to 15 cm. above the cardia. There was no predictable relationship between the size or extent of submucosal esophageal veins and the extent of peri-esophageal veins. Moreover, gross communications between the two venous plexuses appeared sparse and were difficult to demonstrate. Peri-esophageal veins were often more prominent than submucosal veins, and were preferentially filled when the amount of mass injected into the portal vein was small.

Group II

Submucosal esophageal veins were demonstrated in only two of the five cases of congestive heart failure who were injected adequately, while peri-esophageal veins were demonstrated in all five, and were dilated and tortuous in two cases of severe heart failure (Nos. 15 and 16) (Figure 4). Gross anastomotic connections were again demonstrated between the peri-esophageal veins, mediastinal veins, and azygos or hemi-azygos veins (Figure 4). From this venous network, bronchial venules and pleuro-pericardial venules filled in some instances. In no instance did injection mass appear in the pulmonary veins or left atrium.

Group III

In all four of the subjects without hepatic disease or congestive heart failure submucosal esophageal veins were visualized extending 5 to 18 cm. upward from the cardia. Peri-esophageal veins were injected in all except No. 17 in which the amount of mass delivered was small. In some instances, mediastinal, pleuro-pericardial, and azygos veins were injected (Figure 5), but these channels never reached the number or size encountered in Groups I and II.

DISCUSSION

Anatomical considerations

Injection of a mass, which does not penetrate capillary networks, has demonstrated anastomoses of the portal venous bed with the superior vena cava through peri-esophageal, mediastinal, and azygos veins. These porto-caval anastomoses were strikingly more numerous and larger in cases with presumptive prolonged hypertension in either the portal venous system or the caval venous system. Portal hypertension was associated with the most prominent anastomotic channels. Small anastomotic channels between the mediastinal veins and the bronchial venous system were also present in some cases. In two cases of portal hypertension, gross anastomoses between the portal venous system and the pulmonary venous system were demonstrated. Although such direct communications were not seen in the remainder of the present material, they have not been ruled out by the method used.

FIG. 2. DEMONSTRATION OF VARICES IN CIRRHOSIS

Case No. 7. Cirrhosis. Mucosal surface of cardia (below) and esophagus. Submucosal esophageal varices are seen extending upward from the cardia. The arrows point to large peri-esophageal varices.

While the submucosal esophageal veins have been mentioned more prominently in the literature, undoubtedly because of their clinical importance, the presence of peri-esophageal veins and their predominance over submucosal veins in some cases of portal hypertension have been noted by others (6, 10–13). The present material supports the view that the peri-esophageal veins represent more likely paths of porto-caval shunts than the submucosal veins (12, 13).

Anastomoses between the peri-esophageal veins, mediastinal veins, bronchial veins and azygos veins have long been recognized (4, 14, 15), as have been anastomoses between the pulmonary veins and the bronchial veins (4, 5, 8). Liebow (8) has reported a case in which mediastinal vessels communicated directly with a pulmonary vein and the left atrium.

Reports of injections of complete collateral pathways between the portal and the pulmonary venous system are fragmentary. Corning (16) states that gastric veins can be injected from the pulmonary veins without referring to the original work. Butler (6) injected India ink into the superior mesenteric vein of three foeti at term; in two the ink passed from the veins of the stomach into the subepithelial and submucosal veins of the lowermost esophagus, but entered the peri-esophageal veins to a much higher level, reaching the posterior bronchial veins and the azygos veins.

More recently, Schoenmackers and Vieten (13) have published post-mortem portal angiograms of a case of cirrhosis which demonstrate passage of injection mass into pulmonary veins and left atrium, presumably by way of mediastinal veins. Their angiograms resemble radiograms taken of Case 7 in the present series. Blackburn (17) demonstrated a large porto-pulmonary venous anastomosis in a case of extrahepatic portal venous obstruction with portal hypertension and left ventricular hypertrophy and failure, which he attributed to a large porto-pulmonary shunt.

Major anomalous connections between the pulmonary venous system and the portal vein have been reported (18), but appear rare and have usually been associated with other cardiovascular anomalies and attributed to persistence of one or more of the primitive connections between the pulmonary and splanchnic systems of veins.

The fact that anastomotic connections have been demonstrated between the portal venous system and the superior caval system and between the latter and the pulmonary veins in essentially normal individuals suggests that the increased prominence of such anastomoses in disease represents a widening of pre-existing anastomotic channels.

FIG. 3. COMMUNICATION BETWEEN ESOPHAGEAL AND AZYGOS VEINS IN CIRRHOSIS

Case No. 7. Cirrhosis. Mediastinum and posterior aspect of esophagus with prominent peri-esophageal and mediastinal veins draining into the large azygos vein (retracted to the left). A piece of paper (on the right) identifies a vein connecting the mediastinal plexus to a right pulmonary vein.

FIG. 4. DILATED PERI-ESOPHAGEAL VEINS IN CONGESTIVE FAILURE
Case No. 14. Congestive heart failure. Posterior mediastinum and esophagus (retracted to the left) with injected peri-esophageal and mediastinal veins.

Such channels should be demonstrable more frequently with suitable modification of injection technique including further limitation of the vascular bed and direct injection into the mediastinal veins or the left atrium.

Physiological considerations

Anatomical demonstration of collateral vascular channels by means of post-mortem injection does not necessarily prove that such pathways are ever functional, nor does it yield any information about the direction of possible blood flow. According to Butler (6) peri-esophageal veins are without valves, except at their entry into the azygos vein. Valves also have been demonstrated in the azygos vein at its entry into the superior vena cava. No valves are known to exist in mediastinal and bronchial veins, or in pulmonary veins. With the reservation that nothing is known about possible veno-motor activity in this region, consideration may now be given to the relative pressure relationships in the three venous beds concerned: the superior caval, the portal, and the pulmonary. Normally, the mean pressures in the pulmonary venous and portal venous systems are about equal (19, 20). Collateral flow is therefore unlikely although not impossible, since significant differences in pressure might arise during the respiratory cycle. While pulmonary venous pressure decreases with inspiration, McMichael (21) has

shown that in the cat portal venous pressure rises with inspiration.

In patients with cirrhosis of the liver and portal hypertension, portal venous pressures have been found to range from 16 to 35 mm. Hg, with an average of 22 mm. Hg (20). Assuming normal pulmonary venous pressure in such patients, a porto-pulmonary shunt is conceivable, especially if an increase in caval venous pressure should develop.

In patients with mitral stenosis or with left heart failure due to aortic valvular lesions, catheterization of the left heart has shown that pulmonary venous pressure may reach values comparable to those prevailing in the portal vein in cirrhosis (22). In such patients pulmo-caval and pulmo-portal shunts may conceivably exist.

The pressure in the pulmonary venous bed is probably higher than the pressure in the intrathoracic superior caval bed throughout most of the respiratory cycle (23). This relationship should favor drainage of the bronchial veins into the azygos system; yet the bronchial veins are generally thought to drain mainly into the pulmonary veins (24). However, in patients with mitral stenosis and pulmonary venous hypertension, Ferguson, Kobilak and Deitrick (25) found submucosal bronchial veins dilated and postulated that blood flow might be reversed, the bronchial venous system acting in effect to decompress the

FIG. 5. VEINS OF THE NORMAL POSTERIOR MEDIASTINUM

Case No. 19. Normal liver. No heart disease. Posterior mediastinal dissection showing injected veins between the esophagus (right) and the aorta (left).

over-distended pulmonary venous system into the superior caval system.

Anastomotic pathways between the portal venous system and the pulmonary venous system have been demonstrated in patients with cirrhosis. With the pressure gradients known to exist between these two vascular beds, the venous admixture responsible for the unsaturation of arterial blood in these patients (3) may be due, at least in part, to porto-pulmonary shunts. Such shunts could account for the otherwise unexplained finding of Faloon, Auchincloss, Eich and Gilbert (26) that the ammonia level of arterial blood may exceed that of mixed venous blood in cirrhotic patients. They may possibly play a role in metastatic spread of infection or tumor. Further physiological implications will be discussed elsewhere (3).

Clinical considerations

The variability in presence and degree of esophageal varices in individual cirrhotic patients has led to much speculation. The present material suggests that the anatomical variation in the esophageal venous pattern determines the location and extent of varices in any given case.

The prominence of submucosal esophageal veins in two patients with heart failure is of interest in view of the clinical (27) and pathological (28) demonstration of esophageal varices in patients with heart failure. Increased right atrial and caval pressure in right heart failure is accompanied by an increase in portal venous pressure to or slightly above the level of right atrial pressure (29, 30). Thus it is not surprising that collateral channels between the portal and caval systems may be dilated. The direction of blood flow, if any, remains in the realm of speculation. It also remains a matter of conjecture whether the pathways discussed would permit blood to flow from the caval into the pulmonary venous system in pure right heart failure.

While prominence of the roentgenographic shadow of the azygos vein has been noted repeatedly in patients with congestive heart failure (31–33), only one report of prominence of this shadow in patients with portal hypertension (34) has come to the attention of the present authors. In 11 of the present cases, chest roentgenograms taken during life were available and technically satisfactory for review. In five cases of cirrhosis (Nos. 2, 4, 7, 8 and 10) and in four cases of congestive heart failure (Nos. 11, 13, 15 and 16) the shadow of the azygos vein was enlarged according to the criteria of Fleischner and Udis (33); it was normal in two of the control cases (Nos. 17 and 20). Enlargement of the azygos vein presumably reflects caval venous hypertension, increased blood volume, or increased azygos flow from portal venous collaterals. Thus roentgenographic demonstration of an enlarged azygos vein may have clinical value.

SUMMARY

In order to study thoracic anastomoses of the portal venous system, the portal vein was injected in 20 human cadavers, including 10 with advanced cirrhosis of the liver and six with congestive heart failure.

In all cases of cirrhosis, thoracic porto-caval anastomoses were very prominent: the short gastric and coronary veins anastomosed with both the submucosal esophageal and peri-esophageal venous plexi. Although the submucosal esophageal veins were generally varicose, they showed great variation in their extent above the cardia. The peri-esophageal plexus, on the other hand, was consistently dilated and anastomosed freely with mediastinal, pleuro-pericardial and azygos veins. In four cirrhotics the mediastinal venous plexus showed anastomoses with bronchial veins, and in two cases injected material was present in the pulmonary veins and in the left atrium. Thus both porto-caval and porto-pulmonary anastomoses may exist in cirrhosis with portal hypertension.

Similar venous pathways were demonstrated in six cases of heart failure and in four cases having neither cirrhosis nor heart failure, but they were generally less prominent and the pulmonary veins did not fill with mass in these cases.

It is concluded that, in addition to the recognized porto-caval anastomoses, porto-pulmonary anastomoses may exist. When the pressure gradient between portal and pulmonary veins becomes significant, it is conceivable that these anastomoses may act as porto-pulmonary shunts, bypassing the lungs and reducing the oxygen saturation of arterial blood.

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REFERENCES

1. Snell, A. M., The effects of chronic disease of the liver on the composition and physicochemical properties of blood: Changes in serum proteins; reduction in oxygen saturation of the arterial blood. Ann. Int. Med., 1935, **9**, 690.
2. Abelmann, W. H., Verstraeten, J. M., Frank, N. R., McNeeley, W. F., and Kowalski, H. J., The alveolar-arterial oxygen pressure gradient in parenchymatous disease of the liver. Clin. Research Proc., 1954, **2**, 47.
3. Abelmann, W. H., Kramer, G., Gravallese, M. A., Jr., and McNeely, W. F., Increased venous admixture in patients with cirrhosis of the liver and decreased arterial oxygen saturation, In preparation.
4. Zuckerkandl, E., Ueber die Anastomosen der Venae Pulmonales mit den Bronchialvenen und mit dem Mediastinalen Venennetze. Sitzungsb. d. k. Akad. d. Wissensch. Math.-naturw. Cl., Abt. 3, 1882, **84**, 110.
5. Marchand, P., Gilroy, J. C., and Wilson, V. H., An anatomical study of the bronchial vascular system and its variations in disease. Thorax, 1950, **5**, 207.
6. Butler, H., The veins of the oesophagus. Thorax, 1951, **6**, 276.
7. Tobin, C. E., The bronchial arteries and their connections with other vessels in the human lung. Surg., Gynec. & Obst., 1952, **95**, 741.
8. Liebow, A. A., The bronchopulmonary venous collateral circulation with special reference to emphysema. Am. J. Path., 1953, **29**, 251.
9. Schlesinger, M. J., Personal communication, 1954.
10. Saxer, F., Beitraege zur Pathologie des Pfortader-Kreislaufs. Centralbl. f. allg. Path. u. path. Anat., 1902, **13**, 577.
11. McIndoe, A. H., Vascular lesions of portal cirrhosis. Arch. Path., 1928, **5**, 23.
12. Walker, R. M., Portal hypertension. Practitioner, 1949, **162**, 211.
13. Schoenmackers, J., and Vieten, H., Archiv und Atlas der normalen und pathologischen Anatomie in typischen Roentgenbildern. Atlas post-mortaler Angiogramme. Fortschr. Geb. Roentgenstrahlen, 1954, Ergaenzungsbd. **69**, 203.
14. Portal, A., Cours d'Anatomie médicale ou élémens de l' Anatomie de l'homme. Paris, Baudouin, 1803, **4**, 533.
15. Konaschko, P. I., Ueber das System der Anastomosen die die Lungenvenen und den linken Vorhof mit den Venen des grossen Kreislaufs verbinden. Ztschr. Anat., 1929, **89**, 672.
16. Corning, H. K., Lehrbuch der topographischen Anatomie fuer Studierende und Aerzte. Muenchen, J. F. Bergmann, 1942, p. 279.
17. Blackburn, C. R. B., Acquired portal-pulmonary venous anastomosis complicating partial oesophagogastrectomy in a patient with portal hypertension. Thorax, 1956, **11**, 30.
18. Brody, H., Drainage of the pulmonary veins into the right side of the heart. Arch. Path., 1942, **33**, 221.
19. Hellems, H. K., Haynes, F. W., and Dexter, L., Pulmonary "capillary" pressure in man. J. Applied Physiol., 1949, **2**, 24.
20. Paton, A., Reynolds, T. B., and Sherlock, S., Assessment of portal venous hypertension by catheterisation of hepatic vein. Lancet, 1953, **264**, 918.
21. McMichael, J., The portal circulation. I. The action of adrenaline and pituitary pressor extract. J. Physiol., 1932, **75**, 241.
22. Abelmann, W. H., and Hancock, E. W., Unpublished observations, 1955.
23. Opdyke, D. F., and Brecher, G. A., Effect of normal and abnormal changes of intrathoracic pressure on effective right and left atrial pressures. Am. J. Physiol., 1950, **160**, 556.
24. Miller, W. S., The Lung. Springfield, Ill., Charles C Thomas, 1947, pp. 79–88.
25. Ferguson, F. C., Kobilak, R. E., and Deitrick, J. E., Varices of the bronchial veins as a source of hemoptysis in mitral stenosis. Am. Heart J., 1944, **28**, 445.
26. Faloon, W. W., Auchincloss, J. H., Eich, R., and Gilbert, R., Ammonia metabolism in cirrhotic patients with portacaval shunts. J. Clin. Invest., 1956, **35**, 701.
27. Palmer, E. D., and Brick, I. B., Esophageal varices in non-cirrhotic patients. Esophagoscopic study. Am. J. Med., 1954, **17**, 641.
28. Weinberg, T., Observations on the occurrence of varices of the esophagus in routine autopsy material. Am. J. Clin. Path., 1949, **19**, 554.
29. Wren, E. M., The portal venous pressure in organic heart disease. Am. Heart J., 1954, **48**, 929.
30. Taylor, W. J., and Myers, J. D., Occlusive hepatic venous catheterization in the study of the normal liver, cirrhosis of the liver and noncirrhotic portal hypertension. Circulation, 1956, **13**, 368.
31. Durieu, H., and Lequime, J., Aspects radiologiques de la veine azygos au cours de l'insuffisance cardiaque. Arch. d. mal. du coeur, 1938, **31**, 609.
32. Gemignani, V., Il quadro radiologico stratigrafico dell'arco della vena grande azigos. Radiol. med., 1946, **32**, 381.
33. Fleischner, F. G., and Udis, S. W., Dilatation of the azygos vein. A roentgen sign of venous engorgement. Am. J. Roentgenol., 1952, **67**, 569.
34. Grilli, A., Indagine radiologica delle varici esofagee ed aumento dell'ombra della vena azigos nella stasi portale. Radiol. med., 1936, **23**, 165.

STUDIES ON PHENOLIC STEROIDS IN HUMAN SUBJECTS. II. THE METABOLIC FATE AND HEPATO-BILIARY-ENTERIC CIRCULATION OF C^{14}-ESTRONE AND C^{14}-ESTRADIOL IN WOMEN [1]

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It has been known for a number of years that only a small percentage of the metabolites of administered estrone or estradiol can be definitely identified in the urine of human subjects (1–6). At the most, 10 to 20 per cent of the injected estrogen were recovered in the urine either by chemical or biological assays. The presence of estrogens in bile of animals and in the feces of women had been known since 1928 (7, 8), but it was not until the efforts of Cantarow, Pearlman, Paschkis, Rakoff, Hansen and Walking that biliary excretion in the dog was shown to constitute a major route of excretion of estrogens (9–13). Subsequently, these investigators demonstrated substantial estrogenic activity in the bile of pregnant women (14). Interestingly, it was shown that the estrogens in the bile of animals existed in the free form, in contrast to the presence of conjugated steroids in the bile of pregnant women (6, 13). Based on their extensive experiments, these authors advanced the theory that estrogens participate in an enterohepatic circulation.

Significant biliary excretion of estrone labelled with radioactive bromine and estradiol labelled with I^{131} was demonstrated when these isotopes became available (15, 16). Unfortunately, the inclusion of the halogens renders the estrogens biologically inactive and makes the results of the studies difficult to compare with those obtained using endogenous steroids. Furthermore, precise quantification of biliary and urinary estrogen excretion was not possible due to lack of knowledge of all of the estrogen metabolites and imperfections of the methods used for their determination.

The availability of C^{14}-labelled estrone and estradiol has made it possible to study the metabolic fate of these steroids more accurately. Such studies have been performed by Beer and Gallagher (17, 18) in human subjects. They demonstrated that about 65 per cent of the radioactivity of C^{14}-estrone or C^{14}-estradiol were excreted in the urine when these steroids were injected either intramuscularly or intravenously. The authors interpreted their experiments to mean that biliary and intestinal elimination played a minor role in the metabolism of estrogens in human subjects. It should be pointed out that while Beer and Gallagher (17, 18) determined the radioactivity in the feces of some of their subjects, no studies on biliary excretion were performed. No reports have appeared on the clearance of C^{14}-estrogens from the blood, although two reports have indicated rapid clearance of injected large amounts of estrogens (5, 19).

Even though the circulating estrogens have not been definitely identified in human subjects, some evidence has been presented to show that considerable estrogenic activity may be associated with the erythrocytes of blood (20–22) and may exceed the levels present in plasma (20, 21).

In this paper we wish to present data on the distribution of radioactivity in the bile, feces and urine following the intravenous injection of C^{14}-estrone or C^{14}-estradiol in women. The rate of clearance from the blood of the free steroids and their conjugated metabolites will be presented. In addition, the distribution of radioactivity in erythrocytes will be reported. The isolation and identification of the various metabolites are the subjects of separate communications (23).

METHODS AND MATERIALS

One to two μc. of 16-C^{14}-estrone (E_1) or 16-C^{14}-estradiol-17β (E_2) [2] (both 1μc. per 370 μg.) were injected

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[2] C^{14}-estrone and C^{14}-estradiol were purchased from Charles E. Frosst & Co., Montreal, Canada. When the preparations were chromatographed in our laboratory,

TABLE I

Clinical data pertaining to experimental subjects

Subject	Age	Wt.	Steroid	Relation to menstrual cycle	Diagnosis
		Kg.			
M. Su.	27	63.0	E_1	2 days pre-period	Normal
V. S.	21	53.0	E_1	Middle	Normal
P. G.	32	53.0	E_1	Middle	Normal
P. Go.	20	50.0	E_1	7 days post-period	Normal
R. C.	23	47.7	E_1	Middle	Normal
A. D.	24	60.0	E_2	4 days post-period	Normal
M. S.	66	44.5	E_1+ 100 mg. E_1 I.V.	Post-menopause	Sarcoma of leg (pre-op.)
M. P.	44	50.9	E_1	1 day post-period	Carcinoma of breast (post-op.)
E. K.	52	43.3	E_1+ 0.5 Gm. I.M.	Post-menopause	Carcinoma of tongue with metastases
C. M.	56	103.9	E_2	Post-menopause	Carcinoma of breast, chronic pyelonephritis
D. F.	29	60.9	E_2	7 days pre-period	Carcinoma of breast (pre-op.)
J. B.	32	64.0	E_2	Period began 1 day after injection	Carcinoma of breast (pre-op.)
G. D.	58	69.0	E_2	Post-menopause	Leiomyosarcoma, diabetes mellitus
M. B.	39	46.8	E_2	7 days post-period	Carcinoma of breast (post-op.)
M. K.	51	49.1	E_2+ 100 mg. E_2 I.V.	7 days post-period	Carcinoma of breast (post-op.)
B. A.	30	99.4	E_1	Pregnant	Para IV, pregnancy in 8th month
S. R.	25	65.9	E_2	Pregnant	Para III, pregnancy in 9th month
F. M.	39	68.2	E_1	1 day pre-period	T-tube drainage, cholecystectomy for chronic cholecystitis
R. D.	60	78.4	E_1	Post-menopause	T-tube drainage, cholecystectomy for chronic cholecystitis
P. C.	66	78.2	E_1	Post-menopause	T-tube drainage, cholecystectomy for chronic cholecystitis
E. H.	49	60.0	E_2	Post-menopause	T-tube drainage, cholecystectomy for hydrops of gallbladder with small cancer, diabetes mellitus, bilateral oophorectomy and hysterectomy 7 years ago
C. P.	72	46.6	E_2	Post-menopause	T-tube drainage, cholecystectomy for hydrops of gallbladder, hysterectomy 6 months ago, diabetes mellitus

intravenously into 22 women: six normal females, two pregnant women in the last trimester, nine patients with cancer, and five women with bile-fistulas (T-tube drainage). The clinical data related to these subjects are presented in Table I. All the subjects were in good nutritional status and none had evident renal or liver dysfunction or more than a mild anemia (hemoglobin concentrations not lower than 11 Gm. per cent), except where indicated. The C^{14}-estrone or C^{14}-estradiol was dissolved in 1 to 2 ml. of absolute ethanol and then diluted with 30 to 50 ml. of saline, which was injected intravenously over a period of 1 to 2 minutes. In two of the subjects (M.S. and M.K.), intravenous infusions of 100 mg. of the non-radioactive estrogens, dissolved in propylene glycol and human serum albumin (24) and then diluted with physiological saline, were given over a period of one hour. At the end of the infusion, the radioactive steroid was injected intravenously during a period of one minute. One patient (E.K.) was injected intramuscularly, with 500 mg. of an aqueous suspension of crystalline estrone over a period of five days (20 mg. five times daily). On the fourth day, 5 μc. of estrone were mixed with the injection mixture given at noon.

over 99 per cent of the radioactivity, determined in an Actigraph (Nuclear Chicago), were located in the areas corresponding to those of standard compounds.

Except for slight, intermittent nausea and mild swelling and pain in the breasts, none of these three subjects showed any untoward effects from the large doses of estrogens.

All the patients with T-tube drainage of bile had normal liver function tests pre-operatively. The steroids

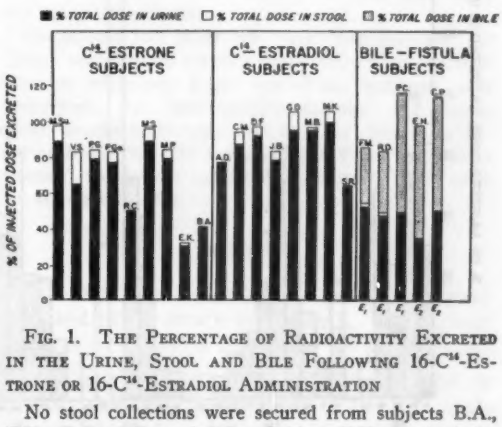

FIG. 1. THE PERCENTAGE OF RADIOACTIVITY EXCRETED IN THE URINE, STOOL AND BILE FOLLOWING 16-C^{14}-ESTRONE OR 16-C^{14}-ESTRADIOL ADMINISTRATION

No stool collections were secured from subjects B.A., E.K., R.C., A.D. and S.R. Subject E.K. received the C^{14}-estrone intramuscularly.

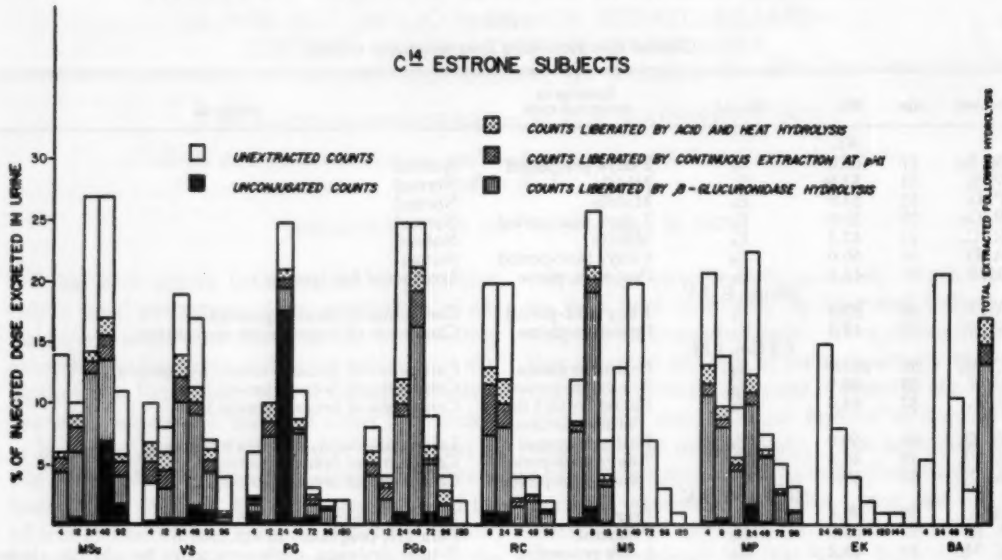

FIG. 2. EXCRETION OF RADIOACTIVITY IN THE URINE FOLLOWING THE INJECTION OF C^{14}-ESTRONE

In this, and in the following figures, the numbers on the abscissa refer to the hour, following the injection of the C^{14}-steroids, when the collection was terminated.

were injected after a patient's condition had become stabilized without fever or other complications, after oral intake of fluids and food had been established, and never sooner than three days post-operatively. Complete biliary collections were assured by a special suction apparatus and by the appearance of acholic stools during and after the period of study.

The methods for collection and processing of urine,

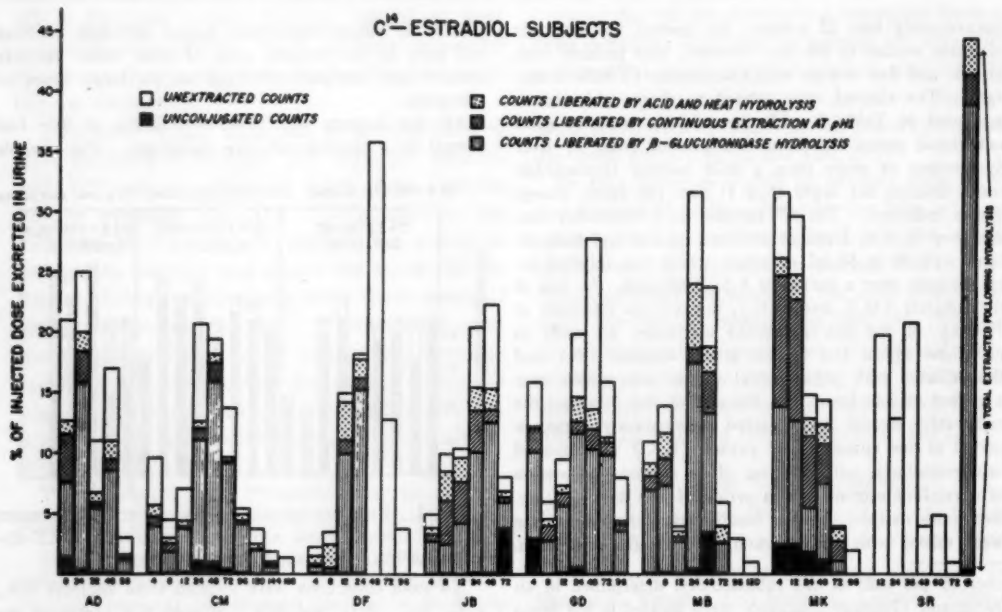

FIG. 3. EXCRETION OF RADIOACTIVITY IN THE URINE FOLLOWING THE INJECTION OF C^{14}-ESTRADIOL

bile and blood, the determination of radioactivity, and the various hydrolytic and extraction procedures have been described in a previous publication (25). One additional procedure was added in the latter part of the study. The determination of total radioactivity in the bile and urine samples was performed in a dual-channel liquid phosphor scintillation counter (TMC Model LP-24). One ml. of urine or 0.2 ml. of bile was added to 10 ml. of a special mixture. The latter consisted of 1,500 ml. of dioxane, 250 ml. of anisole, 250 ml. of ethylene glycol dimethyl ether, 30 Gm. of 2,5-diphenyloxazole and 1 Gm. 1,4-di-(2,4-diphenyloxazyl) benzene. The samples were then centrifuged and let stand in the dark, refrigerated chamber before being counted. An internal standard (1 ml. of a dilute solution of C^{14}-acetic acid in the special mixture) was then added to each sample to determine the extent of "quenching." All samples were counted for a sufficient length of time to register at least 1,000 counts.

The plasma was separated from erythrocytes by centrifugation and the red cells washed twice with large volumes (50 to 150 ml.) of isotonic saline. Total radioactivity was extracted from erythrocytes by addition of absolute ethanol to the cells, centrifugation after standing overnight in the cold, and repetition of the extraction process. The alcohol was evaporated to dryness and the radioactivity determined in the sample.

The plasma protein residues, following the extraction of the plasma with chloroform and then with absolute ethanol, were combined and digested in 0.1 N NaOH over a steam-bath. The solution was then neutralized and extracted with ether.

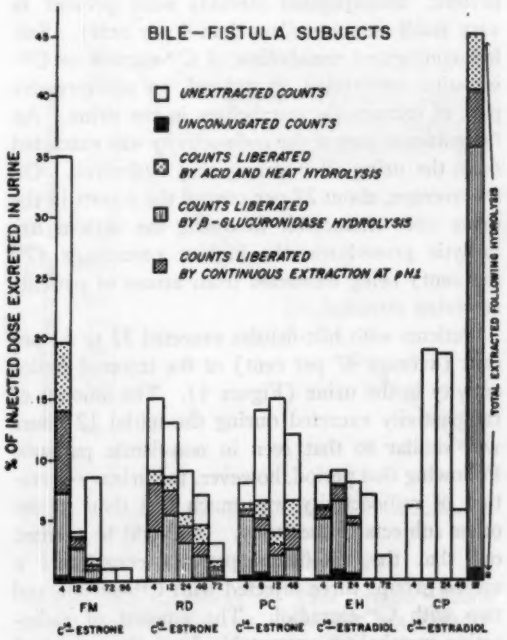

FIG. 4. EXCRETION OF RADIOACTIVITY IN THE URINE OF SUBJECTS WITH BILE-FISTULAS FOLLOWING THE INJECTION OF C^{14}-ESTROGENS

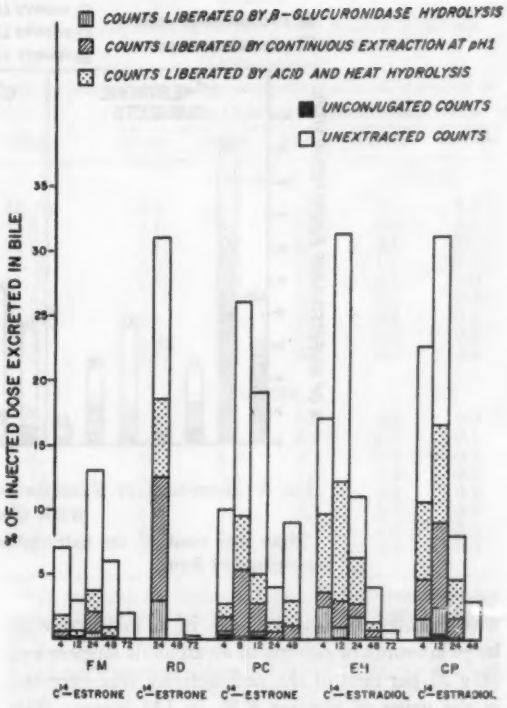

FIG. 5. EXCRETION OF RADIOACTIVITY IN THE BILE OF FIVE SUBJECTS

Note that most of the extractable radioactive metabolites were released following hydrolysis at pH 1 or by hot acid.

Throughout this paper the word "unconjugated," or "free," will refer to steroids which are extractable with ether or chloroform before any of the specimens were hydrolyzed; the "glucuronidate fraction" to steroids which become extractable with ether or chloroform following hydrolysis with β-glucuronidase; and the "sulfate fraction" to steroids extracted continuously with ether for 48 hours at pH 1.

RESULTS

The excretion of injected radioactivity in urine, feces and bile is shown in Figure 1. The amount excreted in the urine during the 96 to 120 hours following intravenous injection of C^{14}-estrone or C^{14}-estradiol ranged (exclusive of the bile-fistula patients) from 41 to 109 per cent (average 80 per cent) of the injected dose. When the C^{14}-estrone

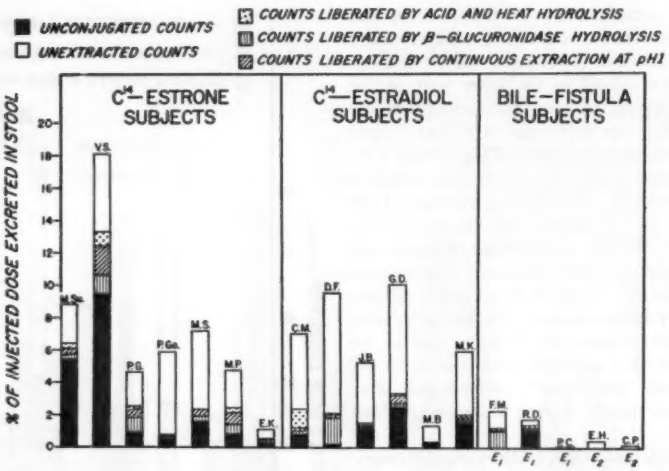

FIG. 6. RADIOACTIVITY EXCRETED IN THE STOOLS OF SUBJECTS INJECTED WITH C^{14}-ESTROGENS

Note that most of the extractable radioactive metabolites occur in the unconjugated form.

was injected intramuscularly in a mixture with large amounts of estrone in an aqueous suspension, only 31 per cent of the radioactivity was excreted in the urine of Subject E.K. in 144 hours. The total radioactivity excreted in the urine of normal subjects did not differ significantly from that of patients with cancer. Of interest was the low excretion of radioactivity in the pregnant women, B.A. and S.R. The rate of excretion of radioactivity in the urine following the administration of C^{14}-estrogens is shown in Figures 2 and 3. A comparison with the rates following the administration of other C^{14}-steroids (25–31) indicates much slower excretion of radioactivity in the urine following C^{14}-estrogen administration than those following other C^{14}-steroids. This was particularly marked following C^{14}-estradiol administration when radioactivity was excreted in the urine as late as 4 to 5 days after intravenous injection.

The urinary excretion of total radioactivity during certain time periods following injection is shown in Figures 2, 3, and 4, as well as the amounts of steroid metabolites extractable following various hydrolytic procedures. It is apparent that a preponderant part of the injected C^{14}-estrone or C^{14}-estradiol is excreted in conjugated form, primarily as the glucuronidate. Except for a few urine samples where, for some reason as yet unknown, large amounts of free metabolites were present, unconjugated steroids were present in very small amounts (less than 4 per cent). Sulfate-conjugated metabolites of C^{14}-estrone or C^{14}-estradiol constituted, in general, an unimpressive part of extractable metabolites in the urine. An insignificant part of the radioactivity was extracted from the urine after strong acid hydrolysis. On the average, about 72 per cent of the counts in the urine were extractable following the various hydrolytic procedures, the highest percentage (79 per cent) being extracted from urines of patients receiving estradiol.

Patients with bile-fistulas excreted 35 to 52 per cent (average 47 per cent) of the injected radioactivity in the urine (Figure 4). The amount of radioactivity excreted during the initial 12 hours was similar to that seen in non-fistula patients. Following that period, however, the urinary excretion of radioactivity was much less than in the other subjects of the study. It should be pointed out that the bile-fistula patients constituted a mixed group: three injected with C^{14}-estrone and two with C^{14}-estradiol. The amount of radioactive metabolites extractable from the urines of the bile-fistula patients was similar to that of the total group, *i.e.*, the largest amount being pres-

ent as the glucuronidate and the smallest as free.

The amount of radioactivity excreted in the bile is shown in Figure 5. The excretion ranged from 31 to 68 per cent (average 52 per cent) and occurred primarily during the first 12 hours. It is interesting to note that most of the extractable metabolites were liberated either with continuous extraction at pH 1 or by hot acid. The amounts of free and glucuronidate metabolites were small. This contrasts with the pattern present in the urine. In addition, a larger percentage of the counts in the bile (59 per cent) was non-extractable after hydrolysis as compared to the urine (36 per cent).

The radioactivity excreted in the stools (Figure 6) of non-fistula patients varied from 1 to 18 per cent (average 7 per cent). In the patients with T-tube drainage, the fecal radioactivity excreted ranged from 0 to 2.2 per cent (average 0.9 per cent). Most of the extractable radioactivity in the stool was present in the free form.

The clearance of free radioactive steroids from the plasma of the injected subjects is shown in Figure 7 and Table II. For the sake of comparison and uniformity, it was assumed that each subject's blood volume constituted 9 per cent of his body weight, with 5 per cent as the plasma volume and 4 per cent as the total red blood cell mass. The results are expressed as the percentage of the injected radioactivity present in the total plasma volume or red cell mass. From Figure 7 it can be seen that the free steroids were cleared from the plasma at two different rates, with half-lives of approximately 20 and 70 minutes. Even though the radioactivity in the free steroid fraction tended on the average to be lower (but not statistically different) following C^{14}-estrone administration than that following C^{14}-estradiol injection, the clearance rates were nearly identical. The presence of a bile-fistula had no effect upon the level of radioactive free steroid. Negligible radioactivity was present in the free steroid fraction eight hours following injection and none could be demonstrated 24 hours later. In Figure 8 and Table III are shown steroid glucuronidate plasma

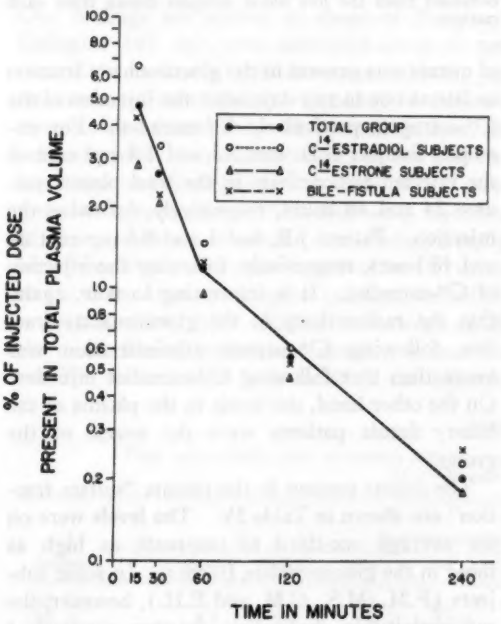

FIG. 7. THE CLEARANCE OF "FREE STEROID" RADIOACTIVITY FROM THE PLASMA FOLLOWING THE INTRAVENOUS INJECTION OF C^{14}-ESTRONE OR C^{14}-ESTRADIOL

Note initial rapid rate and subsequent slower rate of clearance.

TABLE II

Radioactivity levels in the free steroid fraction of plasma following injection of 16-C^{14}-estrone or 16-C^{14}-estradiol *

Subject	% of injected dose present in total plasma volume				
	15 min.	30 min.	60 min.	120 min.	240 min.
			C^{14}-estrone		
M. Su.	3.4	2.4	1.2	0.5	0.2
V. S.	2.5	1.5	0.7	0.4	0.2
P. G.	2.5	2.4	0.9	0.4	0.2
M. S.	5.0	4.3	2.2	1.2	0.2
M. P.	1.8	0.9	0.3	0.1	0.0
B. A.		3.0	0.9	0.4	0.3
F. M.	2.1	1.2	0.5	0.2	0.1
R. D.	2.6	0.8	0.7	0.4	0.0
P. C.	3.2	2.1	0.9	0.5	0.4
			C^{14}-estradiol		
C. M.	5.7	3.1	0.8	0.4	0.0
D. F.	7.8	3.1	0.9	0.6	0.1
J. B.	8.2	4.3	1.5	0.6	0.1
G. D.	6.9	3.5	1.6	0.6	0.4
M. B.	4.9	2.0	1.2	0.4	0.1
M. K.	6.3	3.8	2.0	0.7	0.2
E. H.	9.4	4.5	2.6	0.8	0.6
C. P.	4.5	2.2	1.3	0.8	0.3

* In this, and the following tables, the number of counts present in samples representing values less than 1 were of such magnitude that their counting did not involve a standard error from the mean greater than 5 per cent; in samples representing values less than 0.5 the counting did not involve a standard error from the mean greater than 10 per cent.

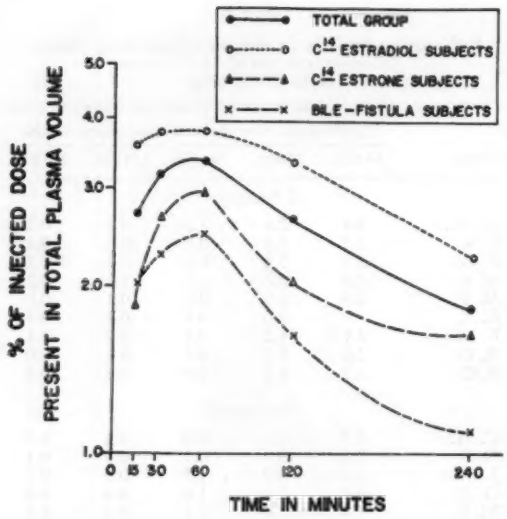

FIG. 8. RADIOACTIVE GLUCURONIDATE STEROID LEVELS IN THE PLASMA FOLLOWING THE ADMINISTRATION OF RADIOACTIVE ESTRONE OR ESTRADIOL INTRAVENOUSLY

levels. Within 30 minutes following injection of the C^{14}-estrogens, the radioactivity in the plasma "glucuronidate fraction" exceeded that present in the unconjugated fraction. Activity declined slowly thereafter. In contrast to the radioactivity in the free steroid fraction, a significant number of counts was present in the glucuronidate fraction as late as one to two days after the injection of the C^{14}-estrogen, particularly C^{14}-estradiol. For example, Subject G.D. had 3.5 and 1.4 per cent of the injected radioactivity in the total plasma volume 24 and 48 hours, respectively, following the injection. Patient J.B. had 1 and 0.5 per cent 24 and 48 hours, respectively, following the injection of C^{14}-estradiol. It is interesting to note, again, that the radioactivity in the glucuronidate fraction following C^{14}-estrone administration was lower than that following C^{14}-estradiol injection. On the other hand, the levels in the plasma of the biliary fistula patients were the lowest of the group.

TABLE III

Radioactivity levels in steroid glucuronidate fraction of plasma following injection of 16-C^{14}-estrone or 16-C^{14}-estradiol

Subject	% of injected dose present in total plasma volume				
	15 min.	30 min.	60 min.	120 min.	240 min.
			C^{14}-estrone		
M. Su.	0.8	1.3	2.4	1.8	1.3
V. S.	1.1	1.5	2.6	2.1	2.3
P. G.	1.4	1.9	2.9	1.6	1.1
M. S.	4.2	5.5	4.5	4.0	1.5
M. P.	1.9	2.5	1.6	0.8	0.4
B. A.		3.6	4.3	2.5	1.8
F. M.	1.4	2.1	1.9	0.8	1.2
R. D.	2.2	3.1	4.1	2.5	0.8
P. C.	1.8	2.2	2.4	2.0	1.6
			C^{14}-estradiol		
C. M.	0.8	0.8	0.9	1.7	2.3
D. F.	2.6	2.7	3.4	5.7	2.2
J. B.	3.8	4.1	4.5	6.0	3.4
G. D.	4.8	6.6	3.4	3.3	4.8
M. P.	4.3	7.8	9.2	3.7	1.3
M. K.	7.2	5.1	4.9	3.4	2.4
E. H.	2.8	2.1	2.0	0.6	0.9
C. P.	2.0	1.9	1.9	2.0	1.0

TABLE IV

Radioactivity levels in steroid sulfate fraction of plasma following injection of 16-C^{14}-estrone or 16-C^{14}-estradiol

Subject	% of injected dose present in total plasma volume				
	15 min.	30 min.	60 min.	120 min.	240 min.
			C^{14}-estrone		
M. Su.	0	0.2	0.4	0.2	0.2
V. S.			0.4	(combined specimens)*	
P. G.			0.8	(combined specimens)	
M. S.	1.4	3.6	2.7	1.6	0.7
M. P.			0.3	(combined specimens)	
B. A.			1.1	(combined specimens)	
F. M.	0.7	1.0	2.2	0.5	1.1
R. D.			2.0	(combined specimens)	
P. C.	0.1	0.2	0.3	0.3	0.2
			C^{14}-estradiol		
C. M.	1.2	0.5	1.3	0.5	0.2
D. F.	1.1	1.1	1.1	1.6	0.5
J. B.	0.4	0.4	1.1	1.4	1.2
G. D.	0.1	0.2	1.1	1.1	1.0
M. P.	1.2	1.4	1.3	1.1	0.5
M. K.	0.2	0.8	0.4	0.3	0.1
E. H.	1.0	1.3	2.4	1.0	0.8
C. P.	0.8	1.0	0.8	0.6	1.2

* The values shown for the "combined specimens" refer to the radioactivity present in the pooled plasma samples obtained from the five blood samples drawn from each patient.

The counts present in the plasma "sulfate fraction" are shown in Table IV. The levels were on the average one-third to one-tenth as high as those in the glucuronidate fraction. In some subjects (F.M., M.S., C.M. and E.H.), however, the radioactivity in the sulfate fraction approached that present in the glucuronidate fraction of these patients.

The radioactivity present in the plasma fractions following hydrolysis by strong acid and heat

was negligible. The radioactivity associated with the red blood cells is shown in Figure 9 and Table V. It should be pointed out that the radioactivity extracted from the erythrocytes represents the *total* number of counts without subsequent hydrolytic procedures. From the data it can be seen that substantial radioactivity may be associated with the red blood cells, although the amount was much lower than that present in the plasma.

The radioactivity present in the protein residues is shown in Table V. Since the calculations are based upon the counts present in the combined protein residues, the results are only a rough approximation of the radioactivity associated with the plasma proteins. In general, the radioactivity associated with the proteins tended to be rather low in comparison with the radioactivity extracted from the plasma.

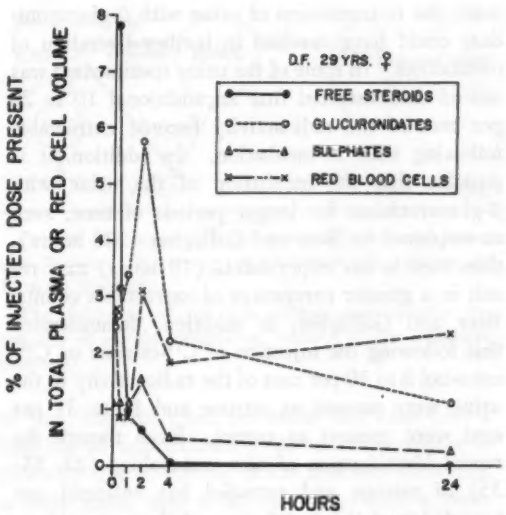

FIG. 9. COMPARISON OF THE TOTAL RADIOACTIVITY LEVELS PRESENT IN THE ERYTHROCYTES TO THOSE PRESENT IN THE PLASMA FREE, GLUCURONIDATE AND SULPHATE FRACTIONS OF SUBJECT D.F. FOLLOWING THE INJECTION OF C^{14}-ESTRADIOL

DISCUSSION

It is apparent from the data presented that the preponderant part of the radioactivity of C^{14}-estrone or C^{14}-estradiol is excreted in the urine. Our findings are similar to those of Beer and Gallagher (17, 18), who recovered about 65 per cent of the radioactivity in the urine following intravenous or intramuscular injection of radioactive estrone or estradiol. These findings contrast markedly with the results obtained in the past using large and unphysiological amounts of nonradioactive estrogens. Thus, following injection of 25 mg. or more of estrone or estradiol, usually less than 20 per cent was recovered in the urine as the three known estrogens (estrone, estradiol, or estriol) (1–6, 32). Without knowledge of all the metabolites of estrone or estradiol, it was impossible to determine how much of the injected steroid appeared in the urine in some other form. The introduction of β-glucuronidase hydrolysis has resulted in the liberation of more extractable free phenolic metabolites following estrogen administration than was possible in the past (17). Labelling of the estrogens with carbon-14 makes it possible to measure recoveries more precisely than with chemical or bioassay techniques.

In our studies about 45 per cent of the radioactivity were extractable from the urine following β-glucuronidase hydrolysis. Somewhat higher values (about 60 per cent) were obtained by Beer and Gallagher (17, 18). It is possible that in our

TABLE V

Total radioactivity levels associated with the erythrocytes and plasma proteins following injection of 16-C^{14}-estrone or 16-C^{14}-estradiol

Subject	% of dose present in total red cell volume									% associated with proteins (combined samples)
	15 min.	30 min.	1 hr.	2 hr.	4 hr.	8 hr.	12 hr.	24 hr.	48 hr.	
M. S.	2.3	0.6	0.3	0.2	0.4	0.3		0.2		0.4
M. K.	2.7	2.4	1.8	0.8	0.7			0.1		0.9
M. B.	0.5	0.3	0.2	0.1	0.1		0.1	0.0		2.1
M. P.	0.5	0.7	0.8	0.8	0.7	0.5				0.5
B. A.		0.2	0.2	0.1	0.1					0.4
G. D.	1.1	0.9	0.4	0.9	0.2	0.6		0.3	0.4	0.5
J. B.	0.9	1.4	0.6	1.6	2.3	3.3		0.4	0.6	1.0
D. F.	2.8	0.7	0.7	3.1	1.9			2.4		

study the re-incubation of urine with β-glucuronidase could have resulted in further liberation of metabolites. In some of the urine specimens it was indeed demonstrated that an additional 10 to 20 per cent of the radioactivity become extractable following such re-incubation. In addition, it is possible that the incubation of the urine with β-glucuronidase for longer periods of time, such as employed by Beer and Gallagher (120 hours), than used in our experiments (48 hours) may result in a greater percentage of extractable counts. Beer and Gallagher, in addition, demonstrated that following the injection of C^{14}-estrone or C^{14}-estradiol 8 to 10 per cent of the radioactivity in the urine were present as estrone and 11 to 31 per cent were present as estriol. Even though the recent identification of new metabolites (23, 33–35) of estrone and estradiol has enlarged our knowledge of the metabolism of these steroids, a significant part of the metabolites of the injected estrogens remains unknown, especially those remaining in the urine following the various hydrolytic and extraction procedures.

Intravenous injection of large amounts of estrone or estradiol with the radioactive steroids did not affect the rate of their excretion. On the other hand, the intramuscular administration of a suspension of crystalline estrone with C^{14}-estrone resulted in only 31 per cent excretion of the radioactivity in the urine over a period of many days. Beer and Gallagher (18) found 65 and 72 per cent of the radioactivity in the urine following the intramuscular injection of C^{14}-estradiol with large amounts of carrier steroid, and 65 per cent following oral ingestion. The discrepancy between the results in our Subject E.K., and those of Beer and Gallagher may be due to the fact that these authors administered the steroid in solution in benzyl alcohol and sesame oil, whereas Subject E.K. was injected with an aqueous suspension of estrone resulting in slower absorption of the steroid.

Radioactivity is excreted much more slowly following C^{14}-estrone or C^{14}-estradiol administration than that following other C^{14}-steroids. Similar observations were made by Beer and Gallagher (17, 18). Thus, following C^{14}-labelled cortisol (27–29), testosterone (25, 26), 11β-hydroxyandrostenedione (31, 36) or corticosterone (30) administration, over one-third of the radioactivity is excreted in the urine in the first four hours and over 60 to 70 per cent in the first 12 hours. On the other hand, following C^{14}-estrone or C^{14}-estradiol administration, only 12 per cent are excreted in the first four hours and about 33 per cent after 12 hours. Following the administration of the former steroids, radioactivity excretion in the urine is usually essentially complete in 48 hours, whereas following C^{14}-estrone or C^{14}-estradiol significant radioactivity was being excreted as late as 96 to 120 hours after the administration of the steroids intravenously.

Even though, in general, the importance of the biliary excretion of estrogens and their enterohepatic circulation had been accepted in the past to be true for animals, *e.g.*, dogs, cows (6, 37), such has not been the case for human subjects. Our data demonstrate, however, that not only was more of the radioactivity of C^{14}-estrone or C^{14}-estradiol excreted in the bile than that seen following the injection of any other known steroid in human subjects (25, 28–30, 38), but in addition more of the radioactivity appeared in the bile than in the urine of three of the bile-fistula subjects. This establishes the importance of biliary excretion in the metabolism of estrogens in women. The importance of the biliary route of excretion of estrogens is in accord with the earlier findings in dogs and in pregnant women (9–14). Similar results were obtained by Albert, Heard, Leblond, and Saffran in animals using I^{131}-labelled estradiol (16). In addition, Twombly and Schoenewaldt (15) demonstrated considerable excretion of radioactivity in the bile of human subjects following the injection of estrone labelled with radioactive bromine. Since the introduction of a halogen into the steroid molecule may affect its metabolism in an unknown fashion, it is difficult to compare these results with those obtained using C^{14}-labelled naturally occuring estrogens.

The large excretion of radioactivity in the bile following C^{14}-estrogen administration is at variance with results obtained by others when excretion of non-labelled estrogens was studied in human subjects (39, 40). This is not surprising since even with labelled C^{14}-estrone and C^{14}-estradiol only about 40 per cent of the radioactivity could be released and extracted from the bile following various hydrolytic procedures. In addition, the estimation of the biliary excretion of

metabolites following non-radioactive administration depended on biological assay or determination of a few known estrogen metabolites. Neither procedure can estimate all the metabolites of estrogens in the bile. Thus, although Rubin, Dorfman and Miller (39) found at least a 30-fold increase in the urinary and an 8- to 10-fold increase in biliary excretions of the three naturally occuring estrogens following the administration of 200 mg. of estradioldipropionate, the total recovery was very small. Somewhat similar findings were observed by Stimmel (40).

In contrast to the findings in the bile of animals, where the estrogens occur in unconjugated form, almost all of the estrogen metabolites present in human bile were in conjugated form. In addition, the percentage of glucuronidate conjugated metabolites was small when compared to similar metabolites in the urine. Most of the extractable metabolites in the bile were released by hydrolysis at pH 1 (with continuous ether extraction) and by hot acid. It should be pointed out that most of the radioactivity in the bile was not extractable following all hydrolytic and extraction procedures.

Since, on the average, only about 7 per cent of the radioactivity appeared in the stool of the non-fistula patients, it is apparent that a considerable reabsorption of estrogen metabolites excreted in the bile must occur in the gastrointestinal tract. In addition, patients with bile-fistulas excreted negligible amounts of radioactivity in the stools. The small amounts that were present may possibly be due to escape of small amounts of bile or to the excretion of steroid metabolites in the gastrointestinal juices. It was interesting to note that most of the extractable radioactivity in the stools was in the free form. Since the steroids occur in the bile in conjugated form, it is possible that the free steroids present in the stool are due to the β-glucuronidase and phenolsulfatase activities of the feces. Such activities have been demonstrated by several observers (28, 41).

Radioactive estrone or estradiol was cleared from the plasma rapidly and at somewhat faster rates than the free steroid clearance following the administration of C^{14}-cortisol or C^{14}-testosterone (25, 29). In addition, there was no significant difference in the clearance of the free steroids among the various subjects studied. The clearance of the free steroids from the plasma occurred

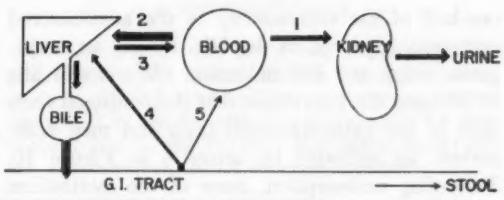

FIG. 10. SCHEMATIC REPRESENTATION OF THE VARIOUS POSSIBLE PATHWAYS OF ESTROGEN METABOLISM IN HUMAN SUBJECTS

The thickness of the arrows indicates roughly the quantity of steroid metabolites involved in the metabolic pathway.

at two different rates, an initial fast rate with a half-life of approximately 20 minutes, which is probably due to mixing of the free steroid within a body pool, and a slower rate, with a half-life of approximately 70 minutes, which probably represents the true metabolic clearance of the free steroid metabolites. The conjugates of the radioactive estrogens reached their peak of radioactivity within 30 minutes and declined slowly thereafter. Following the initial 15 to 30 minutes, the levels of the glucuronidate markedly exceeded those of the free steroids. In addition, no free steroid radioactivity was demonstrable 24 hours following the injection of the radioactive estrogens. On the other hand, substantial radioactivity in the conjugated fractions was demonstrable as late as 48 to 72 hours following the injection. Of importance was the demonstration that the conjugated steroids in the plasma of patients with bile-fistulas tended to be the lowest of the group.

Based on the data of the present experiments and those published in the papers cited, a clearer picture of the metabolic fate and the importance of the hepato-biliary-enteric circulation in the metabolism of administered radioactive estrogens in human subjects can now be advanced. The metabolic pathways of estrogen metabolism are schematically represented in Figure 10. Following injection of radioactive estrone or estradiol a certain amount (usually one-third) is excreted in the urine conjugated with glucuronic acid. This excretion occurred within the first 24 hours following administration and was evident not only in the non-fistula patients but also in the patients with T-tube drainage. About one-third to over

one-half of the radioactivity of the administered estrogens appeared in the bile, mostly as conjugates which are still unknown. We would like to advance the hypothesis that the conjugates are split in the gastrointestinal tract and then reabsorbed, as indicated by arrow 4 in Figure 10. Following reabsorption, some of the metabolites are conjugated again in the liver with glucuronic acid and excreted in the urine, but the preponderant part is reconjugated and re-excreted in the bile to repeat the cycle just described. It is possible that a small amount of metabolites reabsorbed from the gastrointestinal tract escapes conjugation by the liver and enters the circulation to be excreted free in the urine. This is indicated by arrows 5 and 1 in Figure 10. The fact that glucuronidate-conjugated estrogens are not excreted appreciably in the bile, in contrast to the excretion in the urine, lends further support to the hypothesis that the metabolites which appear in the bile must be hydrolyzed in the gastrointestinal tract and reabsorbed as unconjugated steroids, which upon re-entry into the liver are conjugated with glucuronic acid, and are excreted in the urine. An alternate hypothesis would have the steroid metabolites absorbed from the gastrointestinal tract as conjugates, which in the liver under dynamic equilibrium are partially converted to a different type of conjugate.

This continuous recirculation of the metabolites of estrone and estradiol through the liver to the bile and the gastrointestinal tract and the excretion of a certain small percentage of this reabsorbed material in the urine accounts for the slow and delayed excretion in the urine of the radioactivity following the administration of C^{14}-estrone or C^{14}-estradiol. In contrast, steroids which are excreted rapidly in the urine are minimally excreted in the bile. This has been demonstrated to be true for such steroids as cortisol, cortisone, 11-β-hydroxyandrostenedione and testosterone. The re-entry of metabolites through the gastrointestinal tract into the general circulation probably accounts for the persistence of high levels of glucuronidated metabolites in the plasma of the subjects studied. In addition, the low levels of plasma glucuronidated steroids in the T-tube drainage patients are probably due to failure of reabsorption of the steroids present in the bile (Figure 8).

The postulate was advanced by Beer and Gallagher (17, 18) that the slow urinary excretion of radioactivity following C^{14}-estrogen administration, as compared to the excretion following other C^{14}-steroid injections, *e.g.*, testosterone, is related to the binding of the estrogens and their metabolites to plasma and liver proteins (42, 43) with resultant retardation of their renal excretion. In addition, these authors felt that the "relatively minor excretion of estrogen metabolites in the feces is suggestive that biliary and intestinal elimination played but minor roles in the metabolism of the estrogen by human subjects." These authors did not present data, however, on biliary excretion of radioactivity following administration of C^{14}-estrogens. The results of our experiments do not support either of their concepts. We have previously been able to demonstrate that those steroids which are most avidly bound to human plasma proteins have a substantial biliary elimination, whereas those least bound were excreted insignificantly in the bile (44). As in the present study, it was shown that the exclusion of the bile from the gastrointestinal tract results in rapid excretion of radioactivity in the urine. Thus, nearly all of the radioactivity following the injection of C^{14}-estrone or C^{14}-estradiol appeared in the urine and bile within 24 hours. Of the radioactivity excreted in the urine almost 90 per cent appeared in the urine in 24 hours and over 60 per cent in the first 12 hours, in contrast to 60 per cent and 35 per cent, respectively, in the nonfistula group. It should be pointed out that the results of studies indicate avid binding of the estrogenic conjugates to human plasma proteins (44), but the exact role that this binding plays in the rate of urinary excretion of estrogenic metabolites awaits more conclusive and extensive studies.

The appearance of about 7 per cent of radioactivity in the feces, an amount equivalent to that found by Beer and Gallagher in their studies (17, 18), indicates that reabsorption from the gastrointestinal tract of the biliary metabolites may not be complete under normal circumstances, since less than 1 per cent of radioactivity appeared in the feces of patients with bile-fistulas. Since negligible radioactivity is present in the unconjugated fraction in the bile, one would predict that the clearance of the free steroids from patients with

T-tube drainage would resemble that seen in patients without fistulas. That such is the case is demonstrated in Figure 7. The clearance of the free steroids from the plasma of patients with bile-fistulas was almost identical to that of the total group.

The association of radioactivity with the red blood cells would appear to indicate that a significant percentage of endogenous or injected estrone or estradiol and their metabolites are associated with the erythrocytes (20–22, 45). The bulk of the radioactivity was present in the plasma, however. Our findings are at variance with previously published reports in which more estrogenic activity was found in the red blood cells than in the plasma (20, 21). The nature of the metabolites associated with the red blood cells and the effects of these cells on the metabolism of estrogens is a subject which deserves further study.

SUMMARY

C^{14}-estrone and C^{14}-estradiol have been injected intravenously into 22 women. A little over 80 per cent of the administered radioactivity were excreted in the urine during the 96 to 120 hours following the injection of these steroids. On the average, 72 per cent of the counts in the urine were extractable folowing various hydrolytic procedures, with the preponderant part being present in the glucuronidate form. About 50 per cent of the radioactivity administered appeared in the bile, collected through T-tube drainage, mostly as conjugates released by hydrolysis at pH 1 or by the hot acid. Approximately 7 per cent of the radioactivity appeared in the stools of non-fistula patients, indicating gastrointestinal reabsorption of metabolites excreted in the bile. The radioactive steroids were cleared from the plasma rapidly with at least two separate rates having half-lives of approximately 20 and 70 minutes, respectively. The conjugated metabolites of C^{14}-estrone and C^{14}-estradiol reached their peak in the plasma within 15 to 30 minutes following the injection of the steroids and retained high levels for as long as three days. The levels of the conjugated steroids in the plasma of patients with T-tube drainage of bile were the lowest of the group. The significance of the hepato-biliary-enteric circulation of estrogens in relation to the slow urinary excretion of the radioactivity is discussed.

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REFERENCES

1. Smith, G. V., and Smith, O. W., Observations concerning the metabolism of estrogens in women. Am. J. Obst. & Gynec., 1938, **36**, 769.
2. Pearlman, W. H., and Pincus, G., The metabolism of estrone in men. J. Biol. Chem., 1943, **147**, 379
3. Pincus, G., and Pearlman, W. H., Metabolism of estrone in men and non-pregnant women. Endocrinology, 1942, **31**, 507.
4. Heard, R. D. H., and Hoffman, M. M., Steroids IV. The fate in man of injected α-estradiol. J. Biol. Chem., 1941, **141**, 329.
5. Zondek, B., and Black, R., Estrone clearance test in infectious hepatitis. J. Clin. Endocrinol., 1947, **7**, 519.
6. Paschkis, K. E., and Rakoff, A. E., Some aspects of the physiology of estrogenic hormones. Rec. Prog. Hormone Research, 1950, **5**, 115.
7. Gsell-Busse, M. A., Thelykinine in der Galle. Klin. Wchnschr., 1928, **7**, 1606.
8. Dohrn, M., and Faure, W., Uber die Ausscheidung des weiblichen Sexual hormones. Klin. Wchnschr., 1928, **7**, 943.
9. Cantarow, A., Rakoff, A. E., Paschkis, K. E., Hansen, L. P., and Walking, A. A., Excretion of estrogen in bile. Endocrinology, 1942, **31**, 515.
10. Cantarow, A., Rakoff, A. E., Paschkis, K. E., Hansen, L. P., and Walking, A. A., Excretion of exogenous and endogenous estrogen in bile of dogs and humans. Proc. Soc. Exper. Biol. & Med., 1943, **52**, 256.
11. Pearlman, W. H., Paschkis, K. E., Rakoff, A. E., Cantarow, A., Walking, A. A., and Hansen, L. P., A note on the biliary excretion of endogenous estrogens. Endocrinology, 1945, **36**, 284.
12. Pearlman, W. H., Rakoff, A. E., Cantarow, A., and Paschkis, K. E., The isolation of estrone from the bile of pregnant cows. J. Biol. Chem., 1947, **170**, 173.
13. Pearlman, W. H., Rakoff, A. E., Paschkis, K. E., Cantarow, A., and Walking, A. A., The metabolic fate of estrone in bile-fistula dogs. J. Biol. Chem., 1948, **173**, 175.
14. Pearlman, W. H., and Rakoff, A. E., A note on the estrogens in the bile of pregnant women. Endocrinology, 1949, **44**, 199.
15. Twombly, G. H., and Schoenewaldt, E. F., The metabolism of radioactive dibromoestrone in man. Cancer, 1950, **3**, 601.

16. Albert, S., Heard, R. D. H., Leblond, C. P., and Saffran, J., Distribution and metabolism of iodo-α-estradiol labeled with radioactive iodine. J. Biol. Chem., 1949, **177**, 247.
17. Beer, C. T., and Gallagher, T. F., Excretion of estrogen metabolites by humans. I. The fate of small doses of estrone and estradiol-17β. J. Biol. Chem., 1955, **214**, 335.
18. Beer, C. T., and Gallagher, T. F., Excretion of estrogen metabolites by humans. II. The fate of large doses of estradiol-17β after intramuscular and oral administration. J. Biol. Chem., 1955. **214**, 351.
19. Rakoff, A. E., Cantarow, A., Paschkis, K. E., Hansen, L. P., and Walking, A. A., Removal of exogenous estrogens from the circulation. Endocrinology, 1944, **34**, 370.
20. Albrieux, A. S., The distribution of estrogens in the blood of pregnant and non-pregnant women. J. Clin. Endocrinol., 1941, **1**, 893.
21. Albrieux, A. S., Estrogen content of the blood at four-hour intervals. Proc. Soc. Exper. Biol. & Med., 1941, **47**, 380.
22. Bischoff, F., and Katherman, R. E., Distribution of estradiol between serum and red cells. Am. J. Physiol., 1948, **152**, 189.
23. Slaunwhite, W. R., Jr., and Sandberg, A. A., Studies on phenolic steroids in human subjects. I. The conversion of 16-C^{14}-estrone to C^{14}-16-ketoesterone. Arch. Biochem., 1956, **63**, 478.
24. Rothchild, I., A simple method for the preparation of aqueous solutions of steroid hormones for intravenous administration. Endocrinology, 1952, **50**, 583.
25. Sandberg, A. A., and Slaunwhite, W. R., Jr., Metabolism of 4-C^{14}-testosterone in human subjects. I. Distribution in bile, blood, feces and urine. J. Clin. Invest., 1956, **35**, 1331.
26. Fukushima, D. K., Bradlow, H. L., Dobriner, K., and Gallagher, T. F., The fate of testosterone infused intravenously in man. J. Biol. Chem., 1954, **206**, 863.
27. Hellman, L., Bradlow, H. L., Adesman, J., Fukushima, D. K., Kulp, J. L., and Gallagher, T. F., The fate of hydrocortisone-4-C^{14} in man. J. Clin. Invest., 1954, **33**, 1106.
28. Peterson, R. E., Wyngaarden, J. B., Guerra, S. L., Brodie, B. B., and Bunim, J. J., The physiological disposition and metabolic fate of hydrocortisone in man. J. Clin. Invest., 1955, **34**, 1779.
29. Migeon, C. J., Sandberg, A. A., Decker, H. A., Smith, D. F., Paul, A. C., and Samuels, L. T., Metabolism of 4-C^{14}-cortisol in man: Body distribution and rates of conjugation. J. Clin. Endocrinol. & Metab., 1956, **16**, 1137.
30. Migeon, C. J., Sandberg, A. A., Paul, A. C., and Samuels, L. T., Metabolism of 4-C^{14}-corticosterone in man. J. Clin. Endocrinol. & Metab., 1956, **16**, 1291.
31. Bradlow, H. L., Hellman, L., and Gallagher, T. F., Metabolism of 11-hydroxy-Δ^4-androstene-3,17-dione-4-C^{14}. Federation Proc., 1956, **15**, 223.
32. Pearlman, W. H., Pearlman, M. R. J., and Rakoff, A. E., Estrogen metabolism in human pregnancy; a study with the aid of deuterium. J. Biol. Chem., 1954, **209**, 803.
33. Levitz, M., Spitzer, J. R., and Twombly, G. H., The conversion of estradiol-17β-16-C^{14} to radioactive 16-ketoestradiol-17β in man. J. Biol. Chem., 1956, **222**, 981.
34. Marrian, G. F., and Bauld, W. S., The isolation of 16-epioestriol from the urine of pregnant women. Biochem. J., 1955, **59**, 136.
35. Marrian, G. F., Watson, E. J. D., and Panattoni, M., The isolation of a ketonic dihydroxy Kober chromogen from the urine of pregnant women. Biochem. J., 1957, **65**, 12.
36. Sandberg, A. A., and Slaunwhite, W. R., Jr., Unpublished observations.
37. Longwell, B. B., and McKee, F. S., The excretion of estrogens in the bile and urine after the administration of estrone. J. Biol. Chem., 1942, **142**, 757.
38. Hellman, L., Bradlow, H. L., Frazell, E. L., and Gallagher, T. F., Tracer studies of the absorption and fate of steroid hormones in man. J. Clin. Invest., 1956, **35**, 1033.
39. Rubin, B. L., Dorfman, R. I., and Miller, M., Androgen excretion in human bile. Endocrinology, 1952, **51**, 463.
40. Stimmel, B. F., Simultaneous biliary and urinary exogenous estrogen excretion studies in man. Federation Proc., 1951, **10**, 254.
41. Stimmel, B. F., Reduction of radioactive estrone to estradiol by human feces. Federation Proc., 1954, **13**, 305.
42. Szego, C. M., The influence of the liver upon estrogen-protein binding *in vitro*. Endocrinology, 1953, **52**, 669.
43. Riegel, I. L., and Mueller, G. C., Formation of a protein-bound metabolite of estradiol-16-C^{14} by rat liver homogenates. J. Biol. Chem., 1954, **210**, 249.
44. Sandberg, A. A., and Slaunwhite, W. R., Jr., Biliary excretion and protein binding of radioactive steroids in human subjects: Their possible relationship. J. Clin. Endocrinol. & Metab., 1956, **16**, 923.
45. Gray, C. L., and Bischoff, F., Conversion of estrone to estradiol by mammalian red cells. Am. J. Physiol., 1955, **180**, 279.

PATHWAYS OF GLYCOGEN FORMATION IN LIVER AND SKELETAL MUSCLE IN FED AND FASTED RATS[1]

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It is now well established that most animal tissues contain enzymes of a non-glycolytic pathway, the pentose phosphate pathway, in addition to those involved in anaerobic glycolysis (1, 2). Skeletal muscle, however, appears to contain glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase, enzymes of this alternate pathway, in relatively low concentrations.

The relative importance of the glycolytic, pentose phosphate and tricarboxylic acid pathways in the conversion of glucose to CO_2 in liver and in muscle has been the subject of previous *in vitro* studies. Bloom, Stetten and Stetten (3) and Agranoff, Brady and Colodzin (4) found that glucose oxidation proceeded by both glycolytic and non-glycolytic pathways in rat liver slices, while in muscle all the CO_2 arising from glucose could be accounted for via glycolysis. In studies with intact animals, a number of investigators have obtained evidence for the participation of both the glycolytic and pentose phosphate pathways in the formation of glycogen (5–8) and of nucleic acid ribose (8, 9), and in the oxidation of glucose to CO_2 (10) in liver.

The present study was designed to evaluate *in vivo* the relative importance of the various pathways in the formation of glycogen in skeletal muscle and liver of intact rats. The pattern of isotope incorporation into the individual carbon atoms of the glucose of glycogen, when 2-C^{14}-glucose was employed as the tracer source, has been used as an indication of possible differences in liver and muscle glycogen formation. In view of the fact that fasting can markedly deplete the glycogen of liver (11), but not of muscle, and can be associated with definite changes in overall glucose metabolism (12), observations were made on both fasted and fed rats. Striking differences were found between the patterns of tracer incorporation into the glycogen of liver and that of muscle. In addition, the animals' prior dietary status appeared to influence the pathways of glycogen formation.

METHODS

Male rats of the Wistar strain, weighing between 200 and 275 Gm., were fed a nutritionally complete semisynthetic diet (13). In the studies with fasted rats, all food was withdrawn for a 36-hour period prior to isotope administration. All animals were offered water *ad libitum*. One hour prior to the intraperitoneal injection of about 15 μc. of 2-C^{14}-glucose in 0.6 ml. of isotonic saline, each animal was given by stomach tube 1.0 ml. of a 25 per cent glucose solution per 100 Gm. of body weight. The rats were sacrificed by a blow on the head and exsanguinated 6 hours after the administration of the isotope. Liver and skeletal muscle were promptly removed by free-hand dissection. Glycogen was isolated from liver and from muscle by the method of Stetten and Boxer (14). Glucose was obtained by hydrolysis of glycogen with 1 N H_2SO_4 as described by Topper and Hastings (15). Reducing sugar was determined by the method of Somogyi (16). Glucose was degraded by fermentation with *Leuconostoc mesenteroides*, which has been shown by Gunsalus and Gibbs (17) to yield CO_2 from C-1,[2] ethanol from C-2 and C-3, and lactic acid from C-4, C-5 and C-6. The details of the degradation procedure have been described previously (13). All carbon atoms obtained by the degradation of the glucose molecules were converted to barium carbonate and radioactivity assayed at infinite thickness in a windowless flow counter with an absolute counting efficiency of 12 per cent. A sufficient number of counts was recorded to insure a standard error of less than five per cent. The specific activity of the glycogen samples was determined by plating and counting the glycogen as such. Radioactivities are expressed in millimicrocuries per mg. by employing an experimentally determined conversion factor.

The 2-C^{14}-glucose employed in this study was obtained from Dr. H. Isbell, of the National Bureau of Standards.

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[2] The symbol C refers to carbon atom and the number following to the position of the carbon atom in the glucose molecule.

TABLE I

Incorporation of C^{14} into glycogen of liver and muscle following intraperitoneal injection of 2-C^{14}-glucose

Experiment no.	Dietary status	Specific activity*		Ratio L/M
		Liver (L)	Muscle (M)	
1	Fasted	2.43	1.51	1.61
2	Fasted	2.27	1.35	1.68
3	Fasted	3.18	1.94	1.64
4	Fed	0.14	0.89	0.16
5	Fed	0.20	1.50	0.13

* Millimicrocuries per mg. of glycogen.

RESULTS

Incorporation of C^{14}-glucose into glycogen

In the fasted animals, the specific activity of the glycogen of liver exceeded that of muscle, while in fed animals, the muscle glycogen had a higher specific activity than did the liver glycogen (Table I). These findings indicate that a greater fraction of liver glycogen was derived from the injected labeled glucose in the fasted than in the fed animals. On the other hand, the specific activity of muscle glycogen was little affected by the animals' dietary status. These findings are consistent with the well-established fact that fasting depletes the liver of glycogen but has little effect upon the level of the glycogen of muscle.

Pattern of C^{14} incorporation into glycogen of liver

In the experiments with fasted animals, 61.1 to 72.0 per cent of the total activity in the glucose of liver glycogen was in C-2 (Experiments 1 to 3, Table II), while in the studies with fed rats, 74.1 and 79.0 per cent of the total activity was incorporated into C-2 (Experiments 4 and 5, Table III). Degradation of the administered 2-C^{14}-glucose revealed that 99.4 per cent of the total activity was originally in C-2. In all of these experiments, the pattern of C^{14} distribution revealed that isotope initially in C-2 contributed tracer to all the other carbons of glucose. In both the fed and fasted animals, the greatest migration from C-2 was to C-1. The specific activity of C-1 relative to C-2 was greater in the fasted animals than in the fed animals. It may be noted that in each instance C-1 possessed a considerably higher specific activity than C-6 and that this difference, too, was considerably more striking in the experiments with fasted animals. Of the amount of isotope that was randomized from C-2 to the positions other than C-1, a somewhat greater amount was incorporated into C-5 than into C-3, C-4, or C-6.

Pattern of C^{14} incorporation into glycogen of muscle

In the glucose of muscle glycogen isolated from fasted rats, 98.0 to 98.7 per cent of the total isotope incorporated was in C-2 (Experiments 1 to 3, Table II). When fed rats were employed, 90.1 and 93.1 per cent of the total radioactivity incorporated into the glycogen of muscle was in C-2 (Experiments 4 and 5, Table III). In all of the experiments, the randomization of C^{14} from C-2

TABLE II

C^{14} distribution in glucose of glycogen isolated from liver and skeletal muscle of fasted rats following intraperitoneal administration of 2-C^{14}-glucose

Experiment no.	Tissue	Per cent of total activity*					
		C-1	C-2	C-3	C-4	C-5	C-6
1	Liver	13.3	72.0 (20.48)	3.4	3.4	5.0	2.9
	Muscle	0.4	98.7 (7.11)	0.2	0.3	0.2	0.2
2	Liver	16.2	65.4 (5.62)	4.2	4.7	5.6	3.9
	Muscle	0.5	98.0 (1.68)	0.3	0.3	0.4	0.5
3	Liver	19.7	61.1 (10.33)	5.4	4.9	6.9	2.0
	Muscle	0.3	98.8 (10.57)	0.2	0.3	0.3	0.1

* Specific activity of the most radioactive carbon in each compound is indicated in parentheses in millimicrocuries per mg. carbon. The values for specific activity are not corrected for carrier glucose which was added for purposes of degradation. The absolute specific activities of the glucose carbons of liver cannot, therefore, be compared with those of muscle.

TABLE III

C^{14} distribution in glucose of glycogen isolated from liver and skeletal muscle of fed rats following intraperitoneal administration of 2-C^{14}-glucose

Experiment no.	Tissue	Per cent of total activity*					
		C-1	C-2	C-3	C-4	C-5	C-6
4	Liver	8.4	74.1 (0.91)	5.0	4.2	5.0	3.3
	Muscle	3.2	90.1 (1.57)	1.7	1.7	1.7	1.6
5	Liver	5.7	79.0 (0.52)	3.4	4.1	4.5	3.3
	Muscle	2.3	93.1 (7.39)	1.3	1.0	1.1	1.1

* Specific activity of the most radioactive carbon in each compound is indicated in parentheses in millimicrocuries per mg. carbon. The values for specific activity are not corrected for carrier glucose which was added for purposes of degradation. The absolute specific activities of the glucose carbons of liver cannot, therefore, be compared with those of muscle.

to C-1, or to the other carbon atoms of glucose, was much less in muscle glycogen than in liver glycogen.

DISCUSSION

In evaluating the present data in terms of the known intermediary reactions which may be involved in the formation of glycogen in liver and muscle, the following possible reaction sequences need be considered (18–20): 1) 2-C^{14}-glucose may be incorporated directly into the glycogen molecule essentially as an intact 6-carbon unit via glucose-6-phosphate and glucose-1-phosphate, so that the label will be exclusively in C-2. 2) The reversible conversion of glucose-6-phosphate to the triose phosphates with isomerization at the level of dihydroxyacetone phosphate and 3-phosphoglyceraldehyde would yield glucose labeled in C-2 and C-5. 3) Conversion of glucose to CO_2 via glycolysis (Embden-Meyerhof) and the tricarboxylic acid cycle and the subsequent reincorporation of CO_2 into glucose by a reversal of these reactions would lead to randomization of isotope initially in C-2 of glucose to all the other carbon atoms of glucose (21). 4) The oxidative decarboxylation of 2-C^{14}-glucose-6-phosphate to ribulose-5-phosphate by the reactions indicated in Figure 1 would yield 1-C^{14}-pentose phosphate. 1-C^{14}-pentose phosphate may be then converted to 1-C^{14}-hexose phosphate via the transketolase reaction (Figure 2). 5) The reversible reactions catalyzed by the enzymes transketolase and transaldolase would result in 1, 3-C^{14}-hexose phosphate (Figure 3).

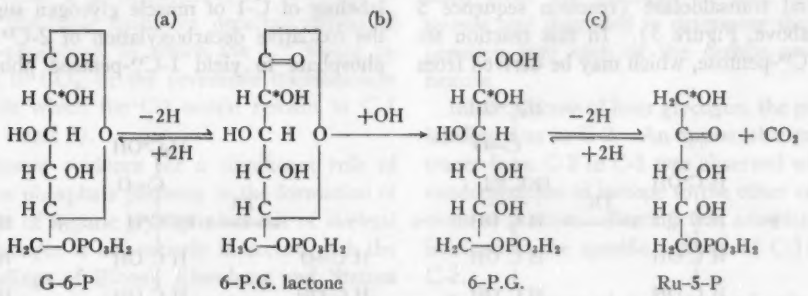

FIG. 1. CONVERSION OF GLUCOSE-6-PHOSPHATE TO RIBULOSE-5-PHOSPHATE

(a) The oxidation of glucose-6-phosphate (G-6-P) to 6-phosphogluconolactone (6-P.G. lactone) involves the reduction of triphosphopyridine nucleotide (TPN) and is catalyzed by the enzyme, glucose-6-phosphate dehydrogenase. (b) The conversion of 6-P.G. lactone to 6-phosphogluconate (6-P.G.) is catalyzed by the enzyme, lactonase. (c) The oxidative decarboxylation of 6-P.G. to ribulose-5-phosphate plus CO_2 involves the reduction of TPN and is catalyzed by the enzyme, 6-phosphogluconic dehydrogenase. The course of the tracer initially in C-2 of glucose is indicated by the asterisk (*) in this Figure and Figures 2 and 3.

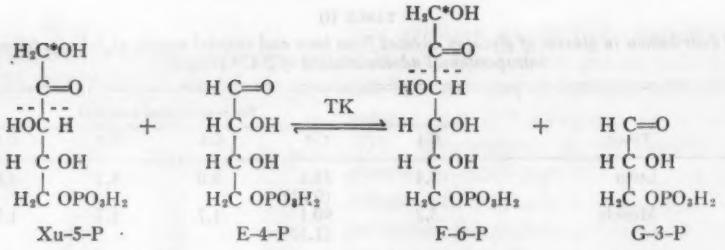

FIG. 2. REACTION CATALYZED BY THE ENZYME, TRANSKETOLASE (TK)

Abbreviations: Xu-5-P, xylulose-5-phosphate; E-4-P, erythrose-4-phosphate; F-6-P, fructose-6-phosphate; G-3-P, glyceraldehyde-3-phosphate. The broken line indicates the group transfer involved in this reaction.

Examination of the pattern of isotope incorporation into liver glycogen, in both fed and fasted rats, reveals that the predominant labeling was in C-2. This finding suggests that the primary pathway of glycogen formation from glucose was by direct incorporation of the administered intact 6-carbon unit (reaction sequence 1 indicated above). However, the striking asymmetric labeling of C-1 is compatible with a significant formation of hexose from pentose via the pentose phosphate pathway (reaction sequences 4 and 5 indicated above, Figures 1 to 3). Fasting appeared to be associated with a greater migration of tracer from C-2 to C-1, which suggests that the pentose phosphate pathway is proportionately more important in the fasting state.

The finding of a higher specific activity of C-1 relative to C-3 in the glucose of hepatic glycogen is not consistent with hexose phosphate formation from pentose phosphate solely by the reaction sequence catalyzed by transketolase and transaldolase (reaction sequence 5 indicated above, Figure 3). In this reaction sequence, 1-C^{14}-pentose, which may be derived from 2-C^{14}-glucose by oxidative decarboxylation, would yield hexose phosphate labeled equally in C-1 and C-3. On the other hand, the reaction catalyzed by transketolase alone (Figure 2) could account for hexose phosphate labeled more heavily in C-1 than in C-3. Transketolase may catalyze an exchange reaction in which the top two carbon atoms of xylulose-5-phosphate equilibrate with the top two carbon atoms of fructose-6-phosphate (Figure 2). In this manner, 1-C^{14}-pentose phosphate could yield 1-C^{14}-hexose phosphate, without necessarily effecting a net synthesis of either sugar.

The randomization of isotope to positions 4 through 6 of glucose, with a predominance of labeling in C-5, is compatible with some contribution to hepatic glycogen formation by the reversible reactions of anaerobic glycolysis and the tricarboxylic acid cycle.

In skeletal muscle, the direct incorporation of administered glucose into glycogen appeared to be the dominant pathway. The minimal asymmetric labeling of C-1 of muscle glycogen suggests that the oxidative decarboxylation of 2-C^{14}-glucose-6-phosphate to yield 1-C^{14}-pentose phosphate oc-

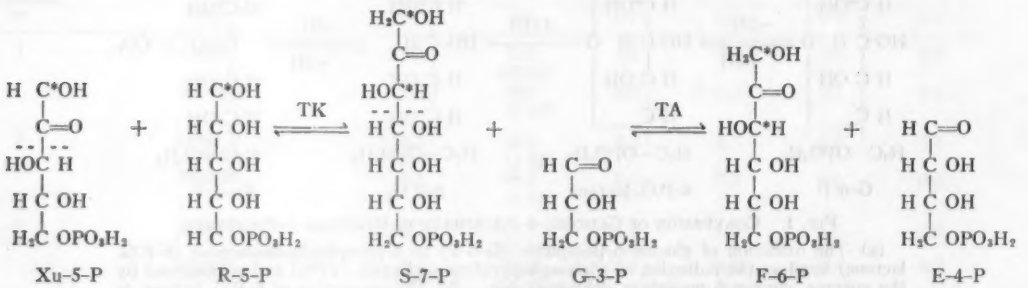

FIG. 3. REACTIONS CATALYZED BY THE ENZYMES, TRANSKETOLASE (TK) AND TRANSALDOLASE (TA)

Abbreviations: R-5-P, ribose-5-phosphate; S-7-P, sedoheptulose-7-phosphate. The broken lines indicate the group transfers involved in these reactions.

curs to a very limited extent, if at all, in skeletal muscle. In the absence of 1-C^{14}-pentose phosphate formation, even though transketolase may be present in skeletal muscle (2), no labeling of C-1 of the glucose of glycogen may be expected. This finding, thus, presents *in vivo* evidence which is compatible with *in vitro* observations that skeletal muscle, in contrast to liver, possesses the enzymes glucose-6-phosphate dehydrogenase and 6-phosphogluconic dehydrogenase in low concentration (1, 2). The possibility exists that the minor amount of tracer incorporated into positions other than C-2 is not a result of muscle metabolism, but reflects the incorporation of glucose originating from the liver. In fed animals, endogenous glucose output might be expected to be greater than in fasted animals and this may be a factor contributing to the somewhat increased randomization of isotope in the glucose of muscle glycogen in the fed as compared with fasted animals.

In previous experiments, Cook and Lorber (22) and Hers (23), employing 1-C^{14}-glucose as the tracer source, found that hepatic glycogen was predominantly labeled in C-1. These authors concluded that glucose is incorporated into hepatic glycogen essentially as an intact 6-carbon unit which does not pass through the pentose phosphate pathway reactions. The failure of these earlier studies to provide evidence for the role of the pentose phosphate pathway in the formation of glucose of hepatic glycogen would seem to be attributable to the use of 1-C^{14}-glucose as the isotope source. 1-C^{14}-glucose could not be expected to detect either a participation of the sequence of reactions involving oxidative decarboxylation of glucose-6-phosphate, in which the C^{14} would be converted to CO_2, or the reversible transketolase reaction, in which the C^{14} would remain in C-1 (Figures 1 and 2).

The present evidence for a significant role of the pentose phosphate pathway in the formation of the glucose of hepatic glycogen but not of skeletal muscle glycogen is not entirely in accord with the recent findings of Bloom, Eisenberg and Stetten (6). These investigators, employing 2-C^{14}-glucose as the tracer source and comparing the isotope distribution in carbons 1 and 6 of glucose of glycogen, found that the asymmetric incorporation of isotope into C-1 was more striking in muscle than in liver glycogen and in three of four experiments there was little or no preferential labeling of C-1 in liver. There are experimental differences between these earlier experiments and the present ones, such as the interval between carrier and isotopic glucose administration to the animal and duration of the experiment, which may account for certain of the disparities between their observations and those of this study. The present findings, however, appear to be more consistent with the observed distribution of the enzymes of the pentose phosphate pathway and the studies of glucose oxidation to CO_2 in liver and muscle.

It should be noted that the present isotope data do not permit precise quantitative estimates of the alternative pathways of glucose formation in terms of net synthesis or exchange reactions. Nevertheless, the data indicate that under the present experimental conditions in the liver of intact rats, the hexose precursor of glycogen is in equilibrium via the transketolase reaction, with pentose derived, at least in part, by oxidative decarboxylation. In skeletal muscle little or no hexose appears to be derived via pentose phosphate formed by oxidative decarboxylation of hexose phosphate. In addition, fasting was associated with a proportionately greater incorporation into liver glycogen but not into muscle glycogen of hexose equilibrated via the oxidative decarboxylation and transketolase reactions.

SUMMARY

Fasted and fed rats were given 2-C^{14}-glucose by intraperitoneal injection; the glucose was isolated from the glycogen of liver and skeletal muscle and degraded to determine the C^{14} incorporation into each of the carbon atoms of the hexose.

In the glucose of liver glycogen, the predominant labeling was in C-2. An appreciable migration of tracer from C-2 to C-1 was observed with a lesser randomization of isotope to the other carbon positions of glucose. Fasting was associated with an increase in the specific activity of C-1 relative to C-2.

In the glucose of skeletal muscle glycogen from fasted rats, tracer was present essentially only in C-2. In the studies with fed animals, somewhat greater randomization of isotope occurred, with C-1 being more heavily labeled than C-3 through C-6.

These data have been interpreted in terms of the known alternative routes of glucose utilization. It is concluded that, in liver, the pentose phosphate pathway contributes to the formation of glycogen glucose and that this is more apparent in the fasted than in the fed animal. In contrast, the glycogen of skeletal muscle appears to be derived almost entirely from glucose by direct incorporation of the intact administered 6-carbon unit, either in the fed or fasted states.

ACKNOWLEDGMENT

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REFERENCES

1. Horecker, B. L., The metabolism of pentose and triose phosphates *in* Phosphorus Metabolism, Vol. 1, W. D. McElroy and B. Glass, Eds. Baltimore, The Johns Hopkins Press, 1951, p. 117.
2. Glock, G. E., and McLean, P., Levels of enzymes of the direct oxidative pathway of carbohydrate metabolism in mammalian tissues and tumours. Biochem. J., 1954, **56**, 171.
3. Bloom, B., Stetten, M. R., and Stetten, D., Evaluation of catabolic pathways of glucose in mammalian systems. J. Biol. Chem., 1953, **204**, 681.
4. Agranoff, B. W., Brady, R. O., and Colodzin, M., Differential conversion of specifically labeled glucose to $C^{14}O_2$. J. Biol. Chem., 1954, **211**, 773.
5. Bernstein, I. A., Lentz, K., Malm, M., Schambye, P., and Wood, H. G., Degradation of glucose-C^{14} with Leuconostoc mesenteroides: alternate pathways and tracer patterns. J. Biol. Chem., 1955, **215**, 137.
6. Bloom, B., Eisenberg, F., and Stetten, D., Occurrence of non-Embden-Meyerhof pathways in the intact rat. J. Biol. Chem., 1956, **222**, 301.
7. Hiatt, H. H., Glycogen formation via the pentose phosphate pathway in mice *in vivo*. J. Biol. Chem., 1957, **224**, 851.
8. Marks, P. A., and Feigelson, P., The biosynthesis of nucleic acid ribose and of glycogen glucose in the rat. J. Biol. Chem., 1957, **226**, 1001.
9. Bernstein, I. A., Synthesis of ribose by the chick. J. Biol. Chem., 1953, **205**, 317.
10. Muntz, J. A., and Murphy, J. R., Pathways of glucose metabolism in rat liver, *in vivo*. Federation Proc., 1955, **14**, 258.
11. Chambers, W. H., Undernutrition and carbohydrate metabolism. Physiol. Rev., 1938, **18**, 248.
12. Lundbaek, K., Metabolic abnormalities in starvation diabetes. Yale J. Biol. & Med., 1948, **20**, 533.
13. Marks, P. A., and Horecker, B. L., Distribution of radioactive carbon dioxide incorporated into rat liver glycogen. J. Biol. Chem., 1956, **218**, 327.
14. Stetten, D., and Boxer, G. E., Studies in carbohydrate metabolism. J. Biol. Chem., 1944, **155**, 231.
15. Topper, Y. J., and Hastings, A. B., A study of the clinical origins of glycogen by the use of C^{14}-labeled carbon dioxide, acetate, and pyruvate. J. Biol. Chem., 1949, **179**, 1255.
16. Somogyi, M., A new reagent for the determination of sugars. J. Biol. Chem., 1945, **160**, 61.
17. Gunsalus, I. C., and Gibbs, M., The heterolactic fermentation. II. Position of C^{14} in the products of glucose dissimilation by Leuconostoc mesenteroides. J. Biol. Chem., 1952, **194**, 871.
18. Horecker, B. L., and Mehler, A. H., Carbohydrate metabolism. Ann. Rev. Biochem., 1955, **24**, 207.
19. Korkes, S., Carbohydrate metabolism. Ann. Rev. Biochem., 1956, **25**, 685.
20. Marks, P. A., A newer pathway of carbohydrate metabolism; the pentose phosphate pathway. Diabetes, 1956, **5**, 276.
21. Wood, H. G., The synthesis of liver glycogen in the rat as an indicator of intermediary metabolism. Cold Spring Harbor Symp. on Quant. Biol., 1948, **13**, 201.
22. Cook, M., and Lorber, V., Conversion of 1-C^{14}-mannose and 1-C^{14}-glucose to liver and muscle glycogen in the intact rat. J. Biol. Chem., 1952, **199**, 1.
23. Hers, H. G., The conversion of fructose-1-C^{14} and sorbitol-1-C^{14} to liver and muscle glycogen in the rat. J. Biol. Chem., 1955, **214**, 373.

THE EFFECT OF HISTAMINE ON SKIN AND DEEP TEMPERATURES IN MAN WITH PARTICULAR REFERENCE TO LIVER TEMPERATURE

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Among the numerous investigations on the effects of histamine on regional temperatures and heat regulation the following reports deserve mention as they deal with problems related to the present investigation:

Dale and Laidlaw (1) reported that a large dose of histamine lowered the body temperature of dogs and guinea-pigs. Harmer and Harris (2) administered a moderate dose of histamine to humans, resulting in an increase of the skin temperature and a rise of rectal temperature. These authors suggested that the opposite effect on the rectal temperature would have seemed more likely. Thiessen and Snell (3), in a series of peptic ulcer cases, found that subsequent to a histamine dose gastric temperature fell whereas oral and rectal temperatures rose. Deutsch, Spitzy and Wohlrab (4) stated that after a dose of histamine the gastric temperature fell to the same extent in achlorhydric cases as in normals. The conclusion was drawn that gastric temperature and secretion are not related to each other. Henning, Demling and Kinzlmeier (5) considered measurement of the gastric temperature a possible method of studying, indirectly, blood flow to the stomach mucosa. They also investigated the reactions of the gastric temperature to certain stimuli (6, 7). The lowering of gastric temperature induced with histamine was interpreted as being due to hyperemia with slow blood flow. Spang, Obrecht and Ey (8) obtained the corresponding effect of histamine on gastric temperautre but did not consider it justifiable to look upon the gastric temperature as representative for blood flow fluctuations. They also stated that the temperature changes in the stomach do not differ from the pattern of other inner organs. Masuda, Ohara and Katsura (9, 10), in a series of experiments on the temperature of the gastrointestinal tract, concluded that gastric temperature and its changes after a histamine dose are related to the blood flow but not to the gastric secretory activity. Benjamin, Wagner, Zeit, Pisciotta, and Ausman (11), in a study of gastric temperature, claimed that in achlorhydria "intragastric temperature was a straight line tracing." These data are entirely contradictory to the findings of other authors (4). Rossi-Espagnet and Torlontano (12) postulated that the decrease in stomach temperature induced with histamine resulted from cooling of the blood circulating in the dilated skin vessels but offered no direct evidence in support of this opinion. Various authors working with animal experiments have previously expressed this view (13, 14).

METHODS

The procedure applied was described in a previous paper (15). Thermocouple units of copper/constantan or chrome-nickel/constantan were used as measuring units with a common reference junction, and the apparatus was built to compensate for variations in room temperature.[1] The constantan wires measured 0.15 mm. in diameter; the size of the copper wires was 0.08 mm. The size of the junction was 0.5×0.3 mm. Junctions and wires were insulated in a nylon tube, the outer diameter of which was 1.55 mm. It was repeatedly checked by means of ice and a heated medium, respectively, so that heat conduction along the thermocouple wires did not distort the results. The measuring units were introduced into the stomach (pyloric region), in the rectal ampulla (immediately above the sphincter), and in the liver. The thermocouple intended for the liver was sterilized for 18 to 24 hours in a 2 per cent solution of benzethonium chloride (NNR) and introduced through a Vim-Silverman-Boecker needle immediately following liver biopsy. The position of the thermocouple in the liver was in several instances controlled by X-ray in two planes. Skin temperature was measured by a thermocouple lightly applied to the center of manubrium. In some cases skin temperature was also measured at the anterior surface of the foreleg. In a few instances the temperature was measured in the right or left hepatic vein, the thermocouple being introduced through a cardiac catheter under fluoroscopic control.

The 40 male patients examined in the present investigation were all admitted to the hospital with alleged or

[1] Equipment built by Ellab, Ltd., Copenhagen.

previously confirmed liver disease. The livers were diagnosed on biopsy as being normal, fatty or cirrhotic but no characteristic differences in temperature pattern between normal and diseased livers have thus far been recognized.

The temperature-measuring equipment was repeatedly checked against a certified mercury thermometer in a water bath of constant temperature, adjusted to different levels between 36 and 38° C. The accuracy of the measuring procedure was found to be ± 0.05° C. As long as the thermocouples mutually yielded entirely uniform figures, small divergences (0.05 to 0.1° C.) from the mercury standard were allowed. The room temperature was constant within 0.5° C. during most of the experiments, and within 1.0° C. in a few. It differed slightly with the seasons of the year (between 18 and 24° C.); in the investigations reported, room temperature variations and changes were not found to exert any influence on the organ temperature patterns examined.

Temperature readings commenced at 9:00 to 9:30 A.M. All subjects had fasted for nine hours and each received a sedative (see below) 30 minutes prior to the liver biopsy. At the hour mentioned, some of the patients offered a relative, or apparent, temperature equilibrium with only small deflections in the temperature curves of the different organs. Other subjects, however, showed at the same hour a tendency for slow fall of temperature in all organs. In these cases it was necessary to wait until the temperature seemed stable enough for experimental procedures. Thus some of the experiments were carried out before noon and others after. In the series of experiments with histamine, reproduced in Figure 1, the smaller and larger dose was given alternatively during morning and afternoon hours in order to reduce as far as possible the influence of spontaneous diurnal temperature shifts during the period in question (9:00 A. M. to 2:00 P.M.).

Drugs used

Histamine. A 0.1 per cent solution of 4-(β-aminoethyl)-imidazol dihydrochloride was used in doses of ½ to 1 ml. given subcutaneously.

Amobarbital. A 0.1 Gm. dose was given orally in each case 30 minutes prior to the liver biopsy.

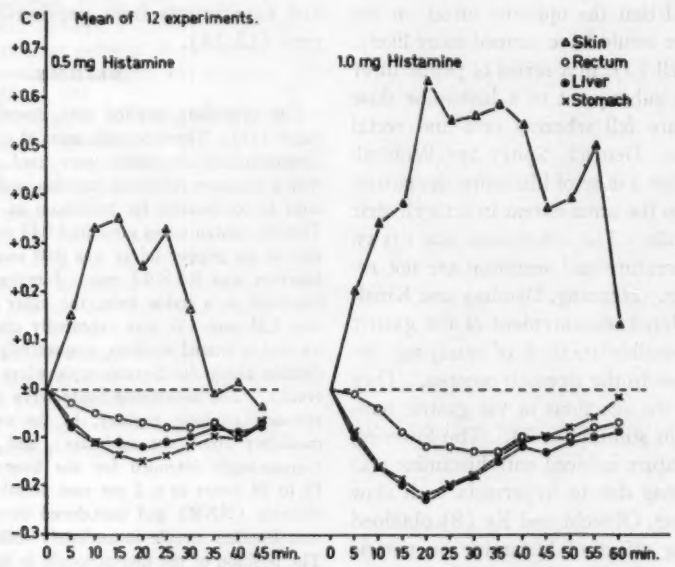

FIG. 1. EFFECT OF HISTAMINE ON SKIN AND DEEP TEMPERATURES

Mean values from 12 cases (see also Table I). Ordinate: change in centigrades from temperature at histamine injection. Abscissa: time in minutes from injection. Absolute temperatures at 0 time:

	0.5 mg. histamine		1.0 mg. histamine	
	Mean	S.E.	Mean	S.E.
Rectum	37.23*	0.067	37.21†	0.070
Stomach	37.11	0.044	37.12	0.071
Liver	36.80*	0.076	36.82†	0.081

* Difference 0.43 ± 0.10° C., P ≅ 0.001.
† Difference 0.39 ± 0.11° C., P < 0.01.

Promethazine. (N-(β-methyl-β-dimethylaminoethyl)-phenothiazine chloride) was used as Lergigan®, brand of Recip Ltd., Stockholm, in doses of 25 mg., given intramuscularly.

Dilatol®. A brand of Troponwerke, Köln, (l-(p-oxyphenyl)-2-(1'-methyl-3'-phenylpropylamino)-propanol-hydrochloride), was given in doses of 5 to 10 mg. subcutaneously.

RESULTS

Organ temperatures during the initial phase of measurement

In those cases where the organ temperatures studied displayed a relatively stable level during the first 30 minutes after applying the thermocouples the experimental arrangements were started without further delay. In cases, however, which showed mobile temperature levels in the shape of a slow fall, the experiments had to be postponed, as mentioned above, until the temperatures leveled off, which occurred within 1 to 2 hours. This fall in temperature did not represent the normal trend of the diurnal rhythm during these hours. In the present material 15 cases were selected to illustrate the initial temperature fall described by Renbourn and Taylor (16). In these 15 cases the temperature at the start of the measurements was set as zero value and the subsequent changes from this value during 90 minutes are reproduced in Figure 2 (mean values, $n = 15$). At 40 minutes the rectal temperature had a value of $-0.13 \pm 0.015°$ C., whereas the corresponding figure for the stomach was $-0.25 \pm 0.029°$ C. The difference between the two organs ($0.12 \pm 0.032°$ C.) has a P value < 0.01 and is thus statistically significant, implying that the lag of the rectal temperature is a real one.

After 90 minutes, or slightly more, a relative stabilization occurred. It is possible that the initial temperature drop in these cases was due to an increased "invisible perspiration" as a sign of stress, but the skin temperatures corresponding to the organ temperatures in Figure 2 did not show any characteristic tendency. In any case, the experimental measures were postponed until the temperature curves seemed to level off. In many cases, however, the curve appeared relatively flat from the beginning and the equilibration period was rather short. In no instance were the experiments started until 30 minutes after applying the measuring equipment in order to let the patient relax after the liver puncture. The measurements were then continued for 3 to 7 hours, *i.e.*, ending at from noon to 4 P.M.

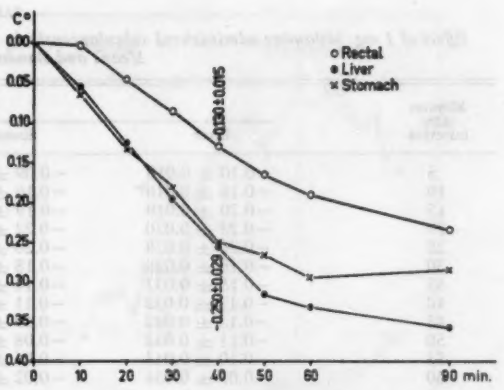

FIG. 2. SPONTANEOUS BEHAVIOR OF ORGAN TEMPERATURES WHEN STARTING THE EXPERIMENT

Mean values from 15 selected cases. Ordinate: centigrade change from first measured value. Abscissa: time in minutes from beginning of experiment.

The difference between rectal and stomach temperatures as indicated at 40 minutes is 0.12° C. ± 0.033 (S.E.), P value less than 0.01.

Mutual interrelationship between organ temperatures under study

In a majority of cases the rectal temperature was highest, the gastric and liver temperatures running a few tenths of a degree centigrade lower. Occasionally, the liver was higher and also the stomach temperature was higher than the two others, but these cases were fairly exceptional. The relationships between the temperature curves of the three organs were relatively constant in one and the same subject but transient crossings sometimes occurred. Inverse relationships between the curves, as well as the mentioned crossings, seemed to lack implication to liver disease.

The effect of histamine

The effect of 0.5 and 1.0 mg. histamine dihydrochloride on the temperature curves of skin (manubrium), rectum, liver and stomach is shown in Figure 1. The centigrade level at the moment of the injection has been set as zero value and the subsequent deflections have been followed. The temperature changes from pre-injection level are also given in Table I. The smaller dose elicits

TABLE I

Effect of 1 mg. histamine administered subcutaneously in terms of changes from temperature levels before the injection. Means and standard errors of 12 cases

Minutes after injection	Temperature change in ° C			
	Liver	Stomach	Rectal	Skin
5	−0.10 ± 0.018	−0.09 ± 0.026	−0.01 ± 0.012	+0.20 ± 0.089
10	−0.16 ± 0.016*	−0.16 ± 0.030	−0.04 ± 0.015*	+0.34 ± 0.117
15	−0.20 ± 0.019	−0.19 ± 0.021†	−0.09 ± 0.024†	+0.38 ± 0.116
20	−0.23 ± 0.020	−0.22 ± 0.023	−0.12 ± 0.026	+0.63 ± 0.156
25	−0.20 ± 0.028	−0.20 ± 0.023	−0.12 ± 0.022	+0.55 ± 0.181
30	−0.18 ± 0.028	−0.18 ± 0.027	−0.13 ± 0.023	+0.56 ± 0.194
35	−0.15 ± 0.032	−0.14 ± 0.032	−0.13 ± 0.024	+0.58 ± 0.225
40	−0.12 ± 0.032	−0.11 ± 0.035	−0.10 ± 0.023	+0.54 ± 0.244
45	−0.13 ± 0.042	−0.10 ± 0.039	−0.10 ± 0.029	+0.37 ± 0.232
50	−0.11 ± 0.032	−0.08 ± 0.051	−0.10 ± 0.026	+0.39 ± 0.206
55	−0.10 ± 0.043	−0.06 ± 0.054	−0.08 ± 0.043	+0.50 ± 0.248
60	−0.09 ± 0.034	−0.02 ± 0.047	−0.07 ± 0.037	+0.13 ± 0.124

* Difference between liver and rectum at 10 minutes = 0.12 ± 0.022° C. ($P < 0.001$).
† Difference between stomach and rectum at 15 minutes = 0.10 ± 0.032° C. ($P = 0.01$).

an average skin temperature rise of 0.35° C., with a concomitant fall of the "deep" temperatures. These display a very uniform pattern, the rectal temperature showing, however, slower fluctuations than the gastric and liver temperatures. No statistical difference was found to exist between the two latter temperature curves. After 45 minutes, all temperatures were back in the vicinity of the pre-injection level. Later on the curves were less representative, being influenced by the diurnal rhythm shift. The experiments were performed on fasting subjects, alternating during morning or afternoon hours. With the larger dose the rise of skin temperature and fall of rectal, hepatic and gastric temperatures were more pronounced, amounting to + 0.6 and − 0.2° C., respectively. In this series, the parallelism between liver and stomach temperatures is even more conspicuous and so is the somewhat tardy character of reaction of rectal temperature. At 20 minutes after

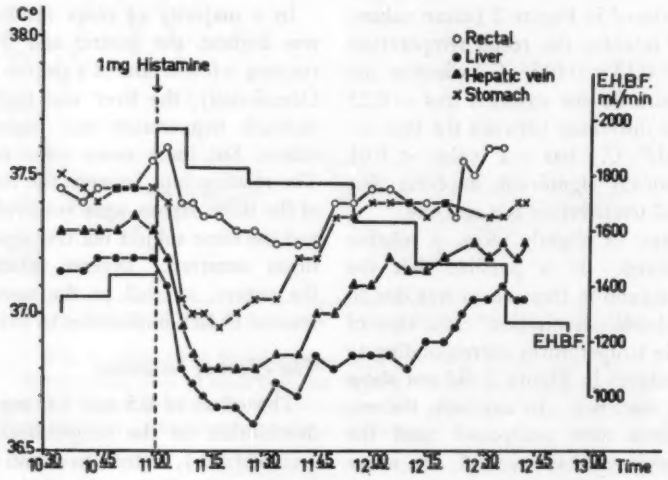

FIG. 3. EFFECT OF 1.0 MG. HISTAMINE

Ordinate: organ temperatures and liver blood flow, respectively. Abscissa: time.

The liver blood flow has been determined with bromosulphalein technique according to Bradley, Ingelfinger, Bradley and Curry (42).

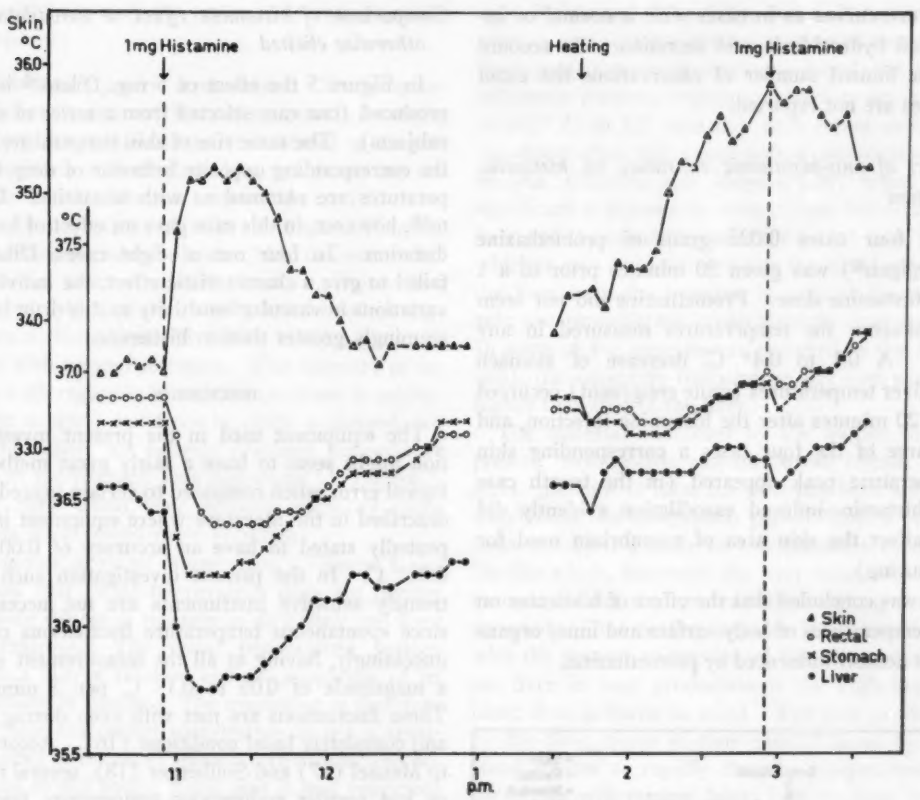

FIG. 4. EFFECT OF HISTAMINE BEFORE AND DURING WARMING OF PATIENT

Ordinate: extreme left scale refers to skin temperature and has been lifted in relation to inner scale, which refers to inner organs. Abscissa: time.

Histamine was administered before and after the patient was put under a heating cradle. The temperature-lowering effect was almost entirely abolished.

the larger dose the difference in temperature between rectum and liver is $0.11 \pm 0.03°$ C., which means that the less pronounced and delayed response of rectal temperature to histamine is statistically significant ($p < 0.01$). In cases where prior to the histamine injection rectal temperature was lower than stomach and/or liver temperatures, the relations were often reversed when the effect of histamine appeared.

In Figure 3 an experiment is reproduced where the temperature in the right hepatic vein was also measured and is seen to behave in the same way as the gastric and the hepatic parenchyma temperatures. The difference between the liver parenchyma and the liver vein in this experiment is at times only $< 0.1°$ C., which closely approaches the margin of the measuring error.

Figure 4 demonstrates the effect of histamine before and during warming of the patient. The subject was put in a semicylindrical radiant heat-cradle, with a total effect of 900 watts, which covered the torso. The extremities were covered with cloth to prevent loss of heat and thus only face and neck were bare. The lowering effect of 1 mg. histamine on rectal, stomach and liver temperatures was almost entirely eliminated.

Effect of histamine in cases of achlorhydria

In two cases of achlorhydria, repeated histamine doses regularly had the same effect on the tem-

perature curves as in cases with a normal or increased hydrochloric acid secretion. On account of the limited number of observations the exact figures are not reported.

Effect of anti-histaminic substance on histamine action

In four cases 0.025 gram of promethazine (Lergigan®) was given 30 minutes prior to a 1 mg. histamine dose. Promethazine did not seem to influence the temperatures measured in any way. A 0.1 to 0.4° C. decrease of stomach and liver temperatures (quite congruent) occurred 5 to 20 minutes after the histamine injection, and in three of the four cases a corresponding skin temperature peak appeared (in the fourth case the histamine-induced vasodilation evidently did not affect the skin area of manubrium used for measuring).

It was concluded that the effect of histamine on the temperatures of body surface and inner organs is not notably influenced by promethazine.

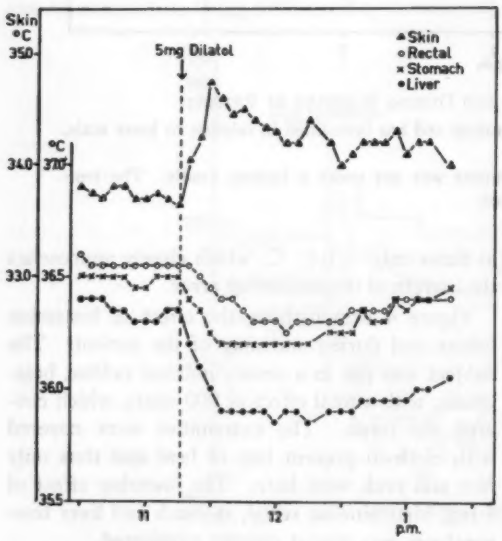

FIG. 5. RISE OF SKIN TEMPERATURE AND FALL OF DEEP TEMPERATURES AFTER ADMINISTRATION OF 5 MG. DILATOL®

Ordinate: as in Figure 4. Abscissa: as in Figure 4. See text.

Comparison of histamine effect to vasodilatation otherwise elicited

In Figure 5 the effect of 5 mg. Dilatol® is reproduced (one case selected from a series of eight subjects). The same rise of skin temperature and the corresponding opposite behavior of deep temperatures are obtained as with histamine. Dilatol®, however, in this case gave an effect of longer duration. In four out of eight cases, Dilatol® failed to give a characteristic effect, the individual variations in vascular sensitivity to this drug being seemingly greater than to histamine.

DISCUSSION

The equipment used in the present investigation might seem to have a fairly great methodological error when compared to certain procedures described in the literature where equipment is repeatedly stated to have an accuracy of 0.005 to 0.01° C. In the present investigation such extremely sensitive instruments are not necessary since spontaneous temperature fluctuations occur unceasingly, having at all the measurement spots a magnitude of 0.05 to 0.1° C. per 5 minutes. These fluctuations are met with even during rest and completely basal conditions (16). According to Menzel (17) and Sollberger (18), several more or less regular endogenous temperature fluctuations occur, having a more frequent periodicity than the well-known diurnal rhythm, and having their different wave lengths superimposed on the latter. Hence, an absolute steady state does not seem to exist. A temperature change of less than 0.05° C. per 5 minutes, therefore, is seldom significant, and an accuracy of less than ± 0.05° C does not constitute an absolute condition when investigating the slow changes which have been studied here.

As mentioned above, Renbourn and Taylor (16) have dealt with the equilibration period when starting temperature measurements, and state, without any attempt to explain the phenomenon, that in general a fall in rectal temperature occurred during this period. During 40 minutes it amounted to − 0.18° C. (S.D. ± 0.3° C.) in Renbourn's quoted series (32 subjects). This figure corresponds fairly well to observations in the present investigation (rectal temperature at 40 minutes = − 0.13° C. (S.E. ± 0.015° C.).

General pattern of the organ temperatures measured

Behavior of gastric temperature. The temperature curves obtained from the stomach decidedly support the opinion that the existence of normal secretion or achlorhydria does not exert any influence on the stomach temperature and its reactions to histamine. Unlike Benjamin, Wagner, Zeit, Pisciotta, and Ausman (11), the author has found, in conformity with Deutsch, Spitzy and Wohlrab (4), that in achlorhydria the fall in temperature elicited with histamine is the same as in cases with normal secretion. The diversity of results with regard to gastric temperature in achlorhydric subjects could not be easily explained, but on the whole an entirely constant temperature level in the stomach (see Reference 11, Figure 3, page 568) seems very unlikely when all other organs show considerable variations.

Behavior of rectal temperature. Figures 1 and 2 show that rectal temperature does not display the same degree of reaction as do gastric and liver temperatures. The rectal temperature pattern shows a slower reaction and the amplitude of its deflections is smaller. The conclusion is therefore justifiable that rectal temperature is not a sensitive index of the rapid temperature fluctuations occurring in the body. Long ago it was suggested that rectal temperature was representative of the critical body temperature but some authors, *e.g.*, Eichna, Berger, Rader and Becker (19), have stressed its inferiority, and Gerbrandy, Snell and Cranston (20) point out that sublingual as well as esophageal temperatures are far better indices of critical tissue temperature.

Mead and Bonmarito (21) performed measurements of rectal temperature gradients at different heights and found that the gastric temperature rose and fell more quickly than rectal temperatures.

Liver temperature. It has been reported that the temperature of the human liver was lower most of the time than the rectal temperature, but that a temporary reversal of the curves could occur, based upon measurings on eight subjects (15). Although this observation seemed inconsistent with some reports based on animal experiments (22–25), it is in agreement with Eichna, Berger, Rader and Becker (19), Horvath, Rubin and Foltz (26), and Sollberger (27) who, in studying the diurnal rhythm of rectal and liver temperatures in chickens, found the mean difference between rectum and liver to be $+ 0.15 \pm 0.02°$ C. in 127 animals. In a recent study on the distribution and regulation of temperature in the rat, Grayson and Mendel (28) found "no significant difference in temperature between the liver and the mesentery near the portal vein, from which it may be concluded that the blood entering the liver from the intestine by way of the portal vein was probably not cooler than the liver itself." In some of the experiments reported in the same work, the mesentery temperature was even slightly higher than that of the liver.

The material examined by the author in the present investigation amounts to 40 human subjects. In some of these the liver has been warmer throughout the observation period, and in others there has been a more or less transient reversal. On the whole, however, the liver temperature of humans did not seem to reach the height generally assumed. This finding appears less inconsistent with the general conception of the role played by the liver in heat production if the high hepatic blood flow is borne in mind. The heat produced by the liver tissue is thus carried away by the blood stream so rapidly that the temperature of the organ will remain fairly low, at least when compared to the rectum, the mucosa of which has greater possibilities of preserving the heat locally produced by the tissues.

It is also possible that the liver, under certain circumstances, actually absorbs heat rather than produces it. This possibility has been put forward previously by Nedzel (29) but does not seem to have been taken into further consideration. According to Miller, Schastnaya and Jutkevich, as cited in an article by Federov and Shur (30) the blood of the portal vein is warmer than the blood of the hepatic vein by 1.5° C. Fedorov and Shur (30) also state that the blood of the portal vein may be warmer than that of the hepatic vein, although this is exceptional.

It might be reasonable to seek an explanation of the findings here reported from another point of view, *i.e.*, why the rectal temperature is particularly high. In the report of Eichna, Berger, Rader and Becker (19), only 5 out of 144 intravascular temperatures measured on 24 subjects

were higher than the rectal temperature of the same subjects. Reference can also be made to Mather, Nahas and Hemingway (31) who mention that "it has been recognized for some time that the rectal temperature in the normal animal is somewhat above that found in the great vessels." Trying to find the key to the considerable rectal heat, Bazett, in a personal communication to Rubin, Horvath and Mellette (32), suggested that the fecal bacterial activity would partly be responsible, but the latter authors found no evidence of this.

Furthermore, it is interesting to observe that according to Klaften (33) and Netter (34) intrauterine temperature seems to lie higher still; according to Bergman (35) it exceeds rectal temperature by 0.3 to 1.0° C., whereby it seems possible that pelvic temperatures in general are high in relation to abdominal ones.

Differences in measuring technique may partly account for contradictory results referred to and certainly it is important that the rectal temperature should be measured at a constant height since it seems to be lower when measured higher up in the rectum (16, 21).

The effect of histamine. Some of the authors quoted above have even yielded results contradictory to those described in this work. Harmer and Harris (2), as well as Thiessen and Snell (3), did not obtain the regular and uniform lowering of deep temperatures but an increase of rectal temperature and a dissociation of deep temperatures. Concerning the first of these reports, the material examined was rather small, and in the second the rectal temperature was measured with a mercury thermometer, which could scarcely be considered satisfactory.

Several authors dealing with the subject have neglected the increased heat loss by cutaneous vasodilatation as an explanation of the lowered "deep" body temperature induced with histamine and have merely suggested changes in blood flow, *e.g.*, in the stomach, as an interpretation of the hypothermic effect of histamine (3, 6, 9, 10).

Results from animal experiments can hardly be accepted as valid for humans. Nonetheless, some of the studies on the mechanism by which histamine exerts its influence on "body" temperature of animals are of interest. Gyermek (13) found that the rectal temperature of rats exposed to an environmental temperature of 20° C. fell from 36.8 to 34.1° C. after injection with histamine. When the room temperature was changed to 30° C. the rectal temperature of the animals rose 1.1° C. The corresponding relations between room temperature and the thermal effect of histamine were observed in rats by Deutsch, Spitzy and Wohlrab (4) and by Fabinyi-Szebehely and Szebehely (14) in rats and mice. These authors thus draw the conclusion that the temperature-lowering effect on the body of histamine at ordinary or low room temperatures is due to increased heat loss through the dilated skin vessels, whereas Packman, Rossi and Harrison (36) suggest that a decreased heat production may partly be responsible for the effect in question.

It is reasonable and logical to assume that in humans, too, the lowering of central body temperatures elicited with histamine is secondary to the vasodilatation in the skin and increased loss of heat from the body surface. The reactions of humans seem to be identical with that of the animals in the quoted experiments, *viz.*, when the environmental temperature is increased and heat loss thus prevented, the fall in temperature is eliminated or decreased. In Figure 4 a minute temperature fall occurs, presumably due to the fact that the head and neck are not enclosed in the cradle or covered. As reported by Bohnenkamp and Ernst (37), the face and neck have high values for heat radiation (calculations based on Stefan-Bolzmann's law) and consequently a considerable loss of heat can occur here.

Further evidence that histamine acts by means of increased heat loss is the fact that its effect can be imitated with other drugs capable of giving a skin vasodilatation, not only Dilatol® as in Figure 5, but also adenosine triphosphoric acid (author's experiments, not reported here). Dilatol® has been studied by Külz and Schneider (38), who found that in rabbits, cats and dogs small doses elicited a strong vasodilatation in skin and muscles. Hensel, Ruef and Golenhofen (39), studying the effect of Dilatol® on blood flow in the muscle and skin of eight human subjects, noticed a marked increase in blood flow in the muscles but practically none in the skin. Measurements in Hensel's investigations were, however, performed on the soles of the feet and presumably the blood vessels of the upper half of the body are more apt to react to this agent. Consequently, the tempera-

ture-lowering effect of Dilatol® demonstrated in Figure 5 may be due to cutaneous vasodilatation as well.

If the temperature-lowering effect of histamine were due to decreased heat production, it would be reasonable to suppose that a decrease of the basal metabolic rate occurred simultaneously. This, however, is not the case, either in animals (13), or in humans, where, *e.g.*, von Euler and Liljestrand (40) obtained a 7 per cent increase of BMR at ordinary room temperature.

In the experiments reported in the present investigation, the liver and hepatic vein temperatures rose and fell simultaneously with the gastric temperature and no evidence was found that the temperature fall in the liver was less or slower when compared with that of the stomach. Kosaka (41), measuring temperatures in the portal and hepatic veins of dogs, found that when the animals were submitted to cooling the fall in temperature in the hepatic vein was much less than in the rectum, allowing the conclusion that heat production in the liver rapidly increased. The results reported above do not support the hypothesis of such a mechanism, as both liver parenchyma and liver vein temperatures reacted in the same way and to the same extent as did the other organ temperatures.

SUMMARY

1. Rectal, gastric, liver and skin temperatures in man have been measured with thermocouples.

2. The lowering effect of histamine was the same on liver and hepatic vein and gastric temperatures, but less marked on the rectal temperature. It corresponded in time to a rise in skin temperature and both effects were proportional to the histamine doses used.

3. The effect of histamine on gastric temperature was the same in achlorhydric patients as in individuals with normal hydrochloric acid secretion.

4. The effect of histamine could be imitated with other agents eliciting cutaneous vasodilatation. Only when a skin temperature rise occurred did the inner organs display a fall in temperature.

5. If cutaneous vasodilatation is induced by heating a subsequent histamine dose has but little effect on the temperatures.

6. It is concluded that the temperature-lowering effect of histamine is merely due to a transient increase of heat radiation from the skin. This interpretation corresponds to results from animal experiments reported by various authors.

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REFERENCES

1. Dale, H. H., and Laidlaw, P. P., Further observations on the action of β-iminazolylethylamine. J. Physiol., 1911, **43**, 182.
2. Harmer, I. M., and Harris, K. E., Observations on the vascular reactions in man in response to histamine. Heart, 1926, **13**, 381.
3. Thiessen, N. W., and Snell, A. M., The variations of intragastric temperature in response to vasodilating agents. Am. J. Physiol., 1933, **105**, 665.
4. Deutsch, E., Spitzy, K. H., and Wohlrab, K., Ueber die Beeinflussung der Magentemperatur durch verschiedene Pharmaka. Arch. internat. pharmacodyn. et de thérap., 1951, **85**, 369.
5. Henning, N., Demling, L., and Kinzlmeier, H., Über ein Gerät zur fortlaufenden, gleichzeitigen Bestimmung wichtiger Magenfunktionen. Klin. Wchnschr., 1951, **29**, 605.
6. Henning, N., Kinzlmeier, H., and Demling, L., Fortlaufende Registrierung verschiedener Magenfunktionen beim Menschen unter dem Einfluss von sekretions- und motilitätsfördernden Substanzen. Ztschr. f. d. ges. exper. Med., 1952, **118**, 177.
7. Demling, L., Kinzlmeier, H., and Henning, N., Experimentelle Untersuchungen über die Wirkung inhibitorischer Pharmaka am menschlichen Magen. Gastroenterologia, 1952, **78**, 269.
8. Spang, K., Obrecht, V., and Ey, W., Über den Wert einer Messung der Temperatur des Magens für die Beurteilung seiner Durchblutung. Klin. Wchnschr., 1952, **30**, 210.
9. Masuda, H., Ohara, M., and Katsura, S., Studies on the temperature of the gastrointestinal tract. Fifth report. Tohoku J. Exper. Med., 1953, **57**, 129.
10. Masuda, H., Ohara, M., and Katsura, S., Studies on the temperature of the gastrointestinal tract. Sixth report. Tohoku J. Exper. Med., 1953, **57**, 137.
11. Benjamin, H. B., Wagner, M., Zeit, W., Pisciotta, A., and Ausman, R. K., Intragastric temperatures in the achlorhydric patient. Surg., Gynec. & Obst., 1955, **100**, 566.
12. Rossi-Espagnet, A., and Torlontano, G., Relazione tra temperatura gastrica, secrezione gastrica e comportamento termico cutaneo e rettale. Boll. Soc. ital. biol. sper., 1955, **31**, 1455.

13. Gyermek, L., Die Wirkung des Histamins auf den Gasstoffwechsel und die Körpertemperatur. Arch. f. exper. Path. u. Pharmakol, 1950, **209**, 456.
14. Fabinyi-Szebehely, M., and Szebehely, J., The influence of antihistamines on the effect of histamine upon body temperature and oxygen consumption in mice and rats. Acta physiol., 1952, **27**, 1.
15. Graf, W., Porjé, I. G., and Allgoth, A.-M., Observations on the temperature of human liver parenchyma. Gastroenterologia, 1955, **83**, 233.
16. Renbourn, E. T., and Taylor, P. F., Body temperature studies: Part II. Rectal and oral as indices of internal temperatures. Presented to the Fifth Commonwealth Defence Conference on Clothing and General Stores. Canada, 1956, p. 28.
17. Menzel, W., Klinische Ziele der Rhythmusforschung. Acta med. Scand. Suppl., 1955, **307**, 107.
18. Sollberger, A., Diurnal changes in biological variability. Acta anat., 1955, **23**, 259.
19. Eichna, L. W., Berger, A. R., Rader, B., and Becker, W. H., Comparison of intracardiac and intravascular temperatures with rectal temperatures in man. J. Clin. Invest., 1951, **30**, 353.
20. Gerbrandy, J., Snell, E. S., and Cranston, W. J., Oral, rectal, and oesophageal temperatures in relation to central temperature control in man. Clin. Sc., 1954, **13**, 615.
21. Mead, J., and Bonmarito, C. L., Reliability of rectal temperatures as an index of internal body temperature. J. Applied Physiol., 1949, **2**, 97.
22. Bernard, C., Recherches expérimentales sur la température animale. Compt. rend. Acad. d. sc., 1856, **43**, 329.
23. Marshak, M. E., Temperature changes in the inner organs caused by warming and cooling the animal. Arch. sc. biol., 1939, **55**, 21.
24. Nedzel, A. J., Temperature changes in the liver in relation to heat and cold on skin. Proc. Soc. Exper. Biol. & Med., 1933, **30**, 689.
25. Dietrick, R. B., and Fritts, J. M., Comparative measurements of temperature in liver, lung, and rectum of the rabbit. Proc. Soc. Exper. Biol. & Med., 1952, **80**, 293.
26. Horvath, S. M., Rubin, A., and Foltz, E. L., Thermal gradients in the vascular system. Am. J. Physiol., 1950, **161**, 316.
27. Sollberger, A., To be published.
28. Grayson, J., and Mendel, D., The distribution and regulation of temperature in the rat. J. Physiol., 1956, **133**, 334.
29. Nedzel, A. J., A study of muscle temperature during bacterial chill. Proc. Soc. Exper. Biol. & Med., 1934, **32**, 279.
30. Fedorov, N. A., and Shur, E. I., The rôle of the viscera in regulating the temperature of the body of an animal under physiological and pathological conditions. Am. J. Physiol., 1942, **137**, 30.
31. Mather, G. W., Nahas, G. G., and Hemingway, A., Temperature changes of pulmonary blood during exposure to cold. Am. J. Physiol., 1953, **173**, 390.
32. Rubin, A., Horvath, S. M., and Mellette, H. C., Effect of fecal bacterial activity on rectal temperature of man. Proc. Soc. Exper. Biol. & Med., 1951, **76**, 410.
33. Klaften, E. M., Utero-thermometry. J. Clin. Endocrinol. & Metab. 1944, **4**, 159.
34. Netter, A., La température cervico-utérine. Ann. d'endocrinol., 1947, **8**, 194.
35. Bergman, P., Studies on ovulation time. Acta obst. et gynec. Scandinav., 1950, **30**, 292.
36. Packman, E. W., Rossi, G. V., and Harrison, J. W. E., The effect of histamine and antihistamines on body temperature. J. Pharm. and Pharmacol., 1953, **5**, 301.
37. Bohnenkamp, H., and Ernst, H. W., Über die Strahlungsverluste des Menschen. Pflüger's Arch. f. d. ges. Physiol., 1931, **228**, 63.
38. Külz, F., and Schneider, M., Über neue gefässerweiternde Sympatlicomimetica. Klin. Wchnschr., 1950, **28**, 535.
39. Hensel, H., Ruef, J., and Golenhofen, K., Die Muskel- und Hautdurchblutung des Menschen bei Einwirkung vasoaktiver Substanzen. Ztschr. Kreislaufforsch., 1954, **43**, 756.
40. von Euler, U., and Liljestrand, G., Die Wirkung von Adrenalin, Sympathol, Tyramin, Ephetonin und Histamin auf Gaswechsel und Kreislauf beim Menschen. Skandinav. Arch. f. Physiol., 1929, **55**, 1.
41. Kosaka, T., On heat production in the liver. J. Orient. Med., 1930, **12**, 19.
42. Bradley, S. E., Ingelfinger, F. J., Bradley, G. P., and Curry, J. J., The estimation of hepatic blood flow in man. J. Clin. Invest., 1945, **24**, 890.

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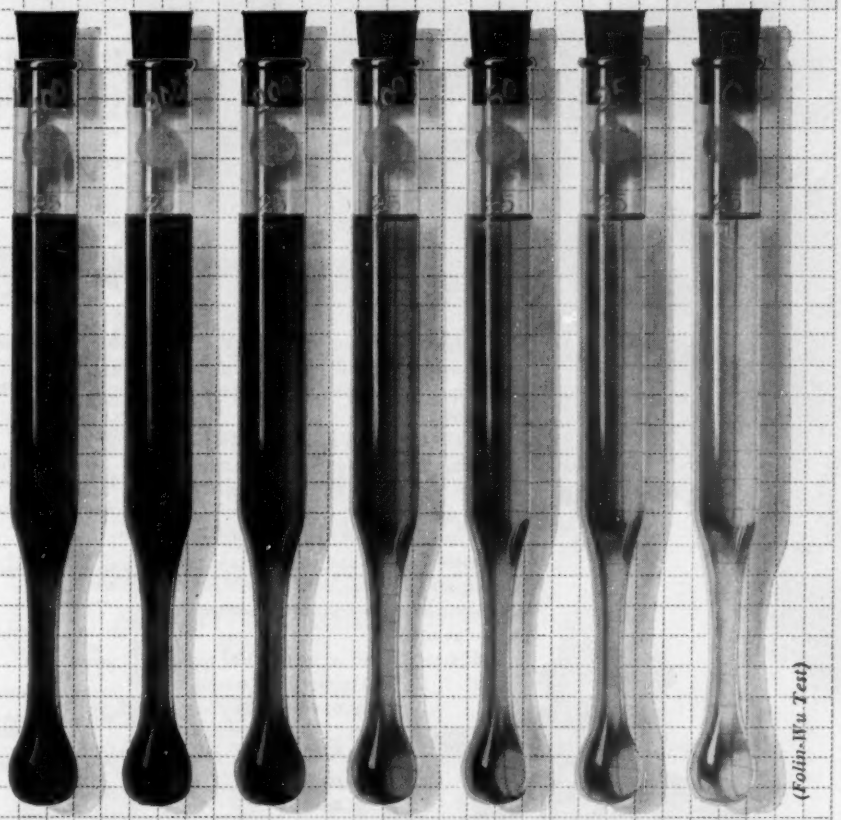

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